Haploid Production in Higher Plant

1* Vijay Kumar Mishra	2Rachna Goswami
Department of Bioscience IIIT Nuzvid	Department of Bioscience IIIT Nuzvid
Rajiv Gandhi University of Knowledge Technologies Nuzvid	Rajiv Gandhi University of Knowledge Technologies Nuzvid
Krishna District Andhra Pradesh, India	Krishna District Andhra Pradesh, India
Email: mishravijay@rgukt.in	Email: rachna.g@rgukt.in
Phone: +91-9177160950	

ABSTRACT: Majority of higher plants are outbreeding, highly heterozygous and undergo a long developmental period before reaching their reproductive stage. Traditional breeding and cross-pollinating procedures are both unpredictable and time-consuming. Haploids are plants with a gametophytic chromosome number and doubled haploids are haploids that have undergone chromosome duplication, represent a particularly attractive biotechnological method to accelerate plant breeding. They can occur either spontaneously or can be induced by modified pollination methods in vivo, or by *in vitro* culture of immature male or female gametophytes. Biotechnologies provide powerful tools for plant breeding, and among these ones, tissue culture, particularly haploid and doubled haploid technology, can effectively help to select superior plants. *In vitro* haploid production is, thus, the most prolific and desirable approach of haploid production. This review describes the range of techniques available for the isolation or induction of haploids by in vivo or by *in vitro*, estimation of haploid lavel either through chromosome counting or flowcytometry and also discourse the value of haploids.

KEY WORDS: Haploidy, Datura innoxia, Hybridization, Chromosome elimination, Ovule culture, Genetic manipulation

1. INTRODUCTION

1.1. Outline of haploidy

The plant life cycle proceeds via alternation of generations of sporophytes and gametophytes. The dominant life form of higher plants is the free-living sporophyte. The sporophyte is the resultant of fertilization of male and female gametes and contains a set of chromosomes from each parent which genomic constitution is *2n*. Cells of the gametophytes carry half the sporophytic set off of chromosomes [*n*], achieved by Chromosome reduction at meiosis means. Haploid plants are sporophytes having only a single set of chromosomes (n; gametophytic number of chromosome). This is in contrast to diploid plants, which contain two sets [2n] of chromosomes [1,2]. Stages in plant life cycle where haploid can occur or be induced shown in Figure 1. The ability to produce haploid plants is a tremendous benefit in genetic, plant breeding, plant physiology and embryology studies. Use of doubled haploids in breeding programs can thus greatly reduce the time required for development of improved cultivars. Heritability studies are simplified, due to haploid plant having only one set of chromosome hence recessive mutation are easily identified. Haploids are sexually sterile and therefore doubling of the chromosomes is required to produce fertile plants which are called doubled haploids or homozygous diploids. Spontaneous haploid production generally produced through parthenogenesis which is originated through ovule androgenesis it means embryo development inside the ovule by the activity of the male nucleus alone [3]. Occurrence of haploid in vivo has been reported in several species, but the frequency is very low [4, 5]. Several strategies and methods have been worked for the production of haploid plant. Kasha and Kao [6] developed haploid in barley through wide hybridization and the subsequent preferential elimination of the wild species chromosomes during early embryogenesis. By this methodology larger number of haploids produced in most of genotypes [7]. This methodology has been successes only in few crops.

In vitro androgenesis via anther-microspore culture is most preferred techniques for obtaining haploids [1, 8] but, *in vitro* gynogenesis via ovary-ovule culture can prove to be a complementary technique in species where anther culture is inaccessible or less productive [9]. The production of haploids via *in vitro* gynogenesis is more tedious, less efficient in comparison to androgenesis [10]. From this it is clear that not only the microspore but, also

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the megaspore of angiosperms can be triggered *in vitro* to undergo sporophytic development. Additionally another techniques *in situ* parthenogenesis (pollen irradiation and chemical treatment) can be employed for generation of haploid plants [11].
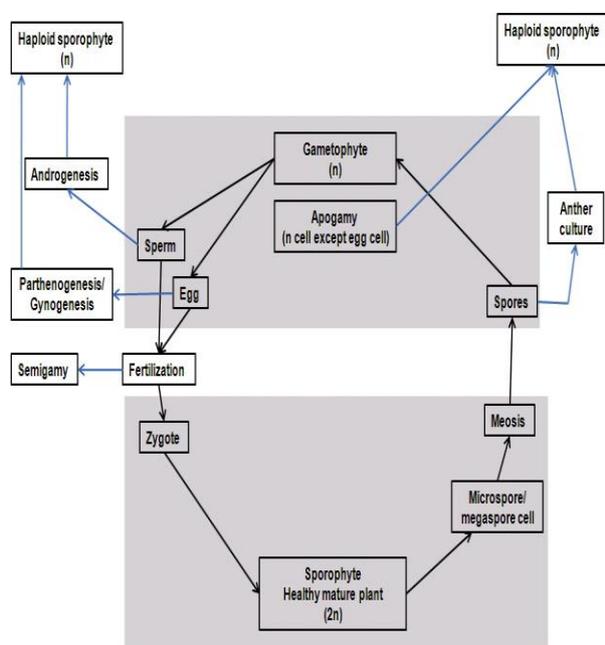

Figure1: **Stages of plant life cycle at that haploid plant can produce**

1.2. History of haploids and double haploid production

The history of doubled haploids [DHs] began with the observation of natural sporophytic haploid in the higher plant, *Datura stramonium* L. (Jimson weed) reported by Bergner in 1921 [13, 14]. This was quickly followed by similar discoveries in other plant species e.g. *Nicotiana tabacum* [14] and *Triticum compactum* [15]. The importance of haploids [and doubled haploids] in breeding and genetics studied was quickly realised and research was started to identify different methods and factors for improvement and high frequency of haploid production [16]. Several methods was identified for production of haploid and doubled haploid including parthenogenesis, pollen irradiation, selecting seed with twin embryos, sparse pollination, alien cytoplasm, wide hybridisation and use of certain genetic stocks [2]. In 1952, Chase first to include haploids in a breeding programme, in this case by inducing haploids in maize via parthenogenesis and then doubling their chromosome complement to produce doubled haploids [17]. A major breakthrough came with the report that haploid plants could be

generated from cultured anthers of *Datura* [18, 19]. It was a advance technique in haploid breeding of higher plants in which development of numerous pollen plantlets in *in vitro* anther cultures of *Datura innoxia* was achieved. Anther cultures of this selected plant, developed from the pollen grains, resulted in embryoids which were haploid [19, 20**]**. This method was tested in many plant species and also established in many species, but with less knowledge of the underlying mechanisms involved. Now a day's anther culture and its derivative technique, isolated microspore culture are preferred methods, though wide crossing and ovule culture are frequently deployed in producing haploids and doubled haploids [21]. A summary of the history of anther culture for haploid production research has been reviewed [22, 23]. These reviews have allowed objective conclusions about the development of male gametophyte *in vitro*. Plant regeneration via anther culture has been reported in more than 250 plants [24]

Due to high frequency of haploid production, attention shifted mainly towards anther culture and culture of female tissues was neglected. The first *in vitro* induced haploid plants of female tissues [gynogenic] origin were achieved by San Noeum [25] in barley. Consequently, Zhu and Wu [26] obtained haploid plants from cultured ovaries of *Triticum aestivum* and *Nicotiana tabacum;* earlier several attempts for induction of haploids by culturing unfertilized ovules were failed [27]. Few unsuccessful and early attempts on culturing unfertilized female ovules are reviewed by Laksmi-Sita [28], Mukhambetzhanov [29] and, more recently, for fruit crops by Germana [30]. After this development of haploid plants via ovary culture in several economically important plant species reports are available, such as, *Zea mays* [31], *Psoralea corylifolia* [32], *Cucurbita pepo* [33], *Guizotia abyssinica* [34], *Cocos nucifera* [35], *Morus alba* [36] etc.

1.3. Current status

The production of haploids through gametic embryogenesis for breeding purposes has been studied by many research groups since the 1970s. There are many published excellent reviews on the production of haploids and doubled haploids including those of 37, 38, 39, 30, 40, 41, , 1, 42, 43, 44, 24, 45, 46, 8, 47, 48, 49]. The doubled haploid techniques have been well reported in a several economically important crop species, including major cereals and cabbages [50]. Haploid production majorly through androgenesis has been reported in

more than 250 plant species, belonging to 100 genera and 40 families [51, 52, 53, 35]. However, in woody species, androgenesis has had only limited success [54, 35]. Several legumes species and woody plants are rather recalcitrant [55, 30, 2009, 1, 56, 57, 58]. List of recent advances in anther culture are also reported [38, 59, 60,8, 47 and 50]. Through wide hybridization method for production of haploids is routinely used in breeding programmes of wheat and other cereal. The general method followed involves a phase of embryo rescue *in vitro*, usually followed by chromosome doubling with colchicine treatment of developed plantlets [24].

2. IN VIVO OCCURRENCE OF HAPLOID PLANTS

Several extensive volumes and reviews are available for the method of production of haploid some of them are Kasha [43], Magoon and Khanna [44], 24, 45, 46, 48, 47]. In conclusion, efforts have been made to obtain haploids from several hundred plant species, although efficient and reproducible DH protocols are few. Some other common and specific information in these and other crop-specific reviews [37, 61, 30, 40, 1, 49], these publications are focused specially on the *in vitro* induction techniques. They do not assess in similar detail the more general occurrence of haploids as rare events in a range of different breeding systems [4].

2.1. Spontaneous haploids

The first haploid angiosperm confirms by cytological proof in 1921 by Dorothy Bergner in *Datura stramonium* [13]. The two haploid plants were obtained among a number of plants of abnormal appearance produced in an attempt to induce chromosomal irregularities by the application of cold as a stimulus. The second confirm haploid was reported in *Nicotiana tabacum* [14]. Gaines and Aase [15], repots first confirmed haploid in cereal species from *Triticum compactum* var. *humboldtii*, a winter variety of the Pacific Northwest. Kimber and Riley [16] were recorded the occurrence of haploids from at least 71 species, representing 39 genera in 16 families of angiosperms, and that number has grown substantially in the decades since, with examples from agropyron [62], alfalfa [63], Citrus [64, 65], peach [66, 67], and *Trillium* [68]. The exact source of haploids in these species, in terms of their embryological origin, is often unknown [38]. Same type of observation were published for gymnosperms

shows several examples of spontaneous haploids [69, 70, 71, 72] that include a unique haploid form of *Thuja plicata* called *Thuja gigantea 'gracilis'* Beissn [73, 74]. Cytological observation of cell, rather than whole plant level, induction of meiosis has been reported in somatic cells [38]. For example, spontaneous reduction in the number of somatic chromosomes was described in the root meristem of *Haplopappus gracilis* by Ames and Mitra [75].

2.2. Hybridization

In native as well as cultivated plants, haploids have been found among the progeny of a huge diversity of crosses between either random or selected parents within the same or different species [38].

2.2.1. Intraspecific hybridization

In few plant species, generation of haploid from diploid material for example in *Haplopappus* [76], and in other cases, diploids have been produced from tetraploids such as in Parthenium [77], Sorghum [78], *Sisymbrium* [79] and alfalfa [80, 81]. Similarly Katayama [82] observed haploid plants in progeny of the allopolyploid *Aegilotriticum*.

Some scientist also reported that crosses between parents with different ploidy levels can produce haploid plant. Asker, [83] identified haploid produced in the progeny of a diploid · tetraploid cross. Same type of case also identified in Citrus plant, here haploid was generated from an interploidy cross between diploids and triploids [84, 85], and haploids of sugar beet [86] were identified by crossing diploid male-sterile plants with green hypocotyls and tetraploid fodder beets homozygous for red hypocotyls [87].

2.2.2. Wide hybridization

Use of wide crosses [interspecific pollinations] between, rather than intraspecific, a species is one of the most successful methods for haploid induction and has been used successfully in many species [50]. Apparao and Varma [88] also got haploid plant from interspaecic hybridization in tobacco. Other recent relevant examples of include haploid generation through wide hybridization technology in *Oenothera* [89], sorghum [90], strawberry [91], *Elymus* [92], chicory [93], pear [94] and *Brassica napa* [95].

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2.2. Chromosome elimination

Selective chromosome elimination has been reported in some wide crosses and frequently results in haploids of one of the parental species. In this process haploid obtained by selective chromosome elimination that follows certain interspecific pollinations. This occurrence was first observed in Barley with crosses between *Hordeum vulgare* and *H. bulbosum* [6] and is now used routinely in wheat and other cereal breeding programmes; haploids were induced in these species following pollination with maize pollen [96]. Uniparental elimination of chromosomes, which occurs in interspecific crosses between *Hordeum vulgare* [cultivated barley] and *H. bulbosum* [bulbous barley grass], is a process which can be used to produce doubled-haploid barley plants in breeding programs [97]. Well describe cytological aspect of chromosome elimination procedure are available [98,99]. Frequency of getting haploid from this procedure depends on several factors such as a range of genetic [100, 101] and experimental variables, as well as the intensity of light at early stages of embryo development [102]. In the addition, the probability of partial hybrids [103], there is an intriguing chances of presence of residual maize DNA in haploids or doubled haploids of wheat [or other species].To date, there has been few investigation of this possibility, and the results are inconsistent [38]. Some evidences are availabe for the transfer of maize-specific DNA [104, 105, 106], whereas no evidence of introgression was reported in two other studies [107, 108].

2.3. Parthenogenesis

Haploid regeneration through un-pollinated female gametophytes is an alternative process for haploid induction. This methodology normally described by the term gynogenesis, or haploid parthenogenesis. Often, the term gynogenesis has been reserved for haploid embryogenesis induced by the presence of a male sperm cell, which does succeed in contributing any genetic material to the embryo; it is used as such in the animal kingdom. In plant terms, gynogenic haploid regeneration is widely used for all haploid induction methods, in which a female gametophyte is used as the origin of haploid cells, regardless of whether a pseudo-fertilization process is involved or not [109]. Frequency of this process is very low. Very less haploid among normal diploid seed offspring [110] and it is very difficult to identify to dectect haploid among them unless specific genetic markers in the pollinators are used for selection,

as exemplified for maize [111] and apple [112]. However, female haploids in seeds of angiosperms are frequent among seeds germinating with two or more seedlings [113]. Occurrence of twin seedlings, and their potential as a source of haploid in angiosperm, has been known many years in several plant species [38] A similar methodology is possible with conifers [114, 115, 116]. Such as, Illies [117] found ten haploid seedlings in the course of an extensive cytological study covering 435 progenies in Picea abies Karst.

With the help of various physical or chemical agents several attempts have been made to increase the frequency of haploids by treating the pollen, prior to pollination. For example, induction of female-derived haploid embryos following pollination with irradiated pollen has been used successfully in many species such as Citrus [118], Pear [119, 94] Apple [120, 121, 122] etc. Chat et al. [123] reported, for the first time, specific variation of pollen irradiation, and the complete uncoupling of the transmission of organelle and nuclear genomes in *Actinidia deliciosa* [kiwifruit], a plant species known for its paternal mode of chloroplast inheritance.

Transmission of nuclear genes from the pollen parent to the progeny was inhibited after the pollen irradiation, but transmission of chloroplast genome was affected. With this it is concluded that plastids can be discharged from the pollen tube into the egg with little or no concomitant transmission of paternal nuclear genes [38].

Heat treatment of pollen is alternative of irradiation of pollen. Heat treated pollen has been used for successfully for induction of haploid chemical treatment of pollen also used successfully such as pollen with toluidine blue in trees [124, 125], maize silks with maleic hydrazide [126] and brassinolide to emasculated stigmas of *Arabidopsis*, *Brassica* and *Tradescantia* [127].

3. IN VITRO METHODOLOGY OF HAPLOID PRODUCTION IN HIGHER PLANT

Through gametic embryogenesis breeding method, since 1970 extensive research has been carried out for haploid production of tree species [128, 30, 53]. Generally, haploids can mainly be produced by two strategies i.e. by regeneration from the male gamete or from the female gamete (Figure 1).

3.1 Haploids from male gametes

In androgenesis immature pollen grains are induced to follow the sporophytic mode of

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development by various physical and chemical stimuli. Haploid production through anther culture is a potentially efficient means to generate homozygous true-breeding progeny lines in plant breeding programs. However, despite considerable efforts, androgenesis in woody plants is difficult, and there are still many recalcitrant species or genotypes [129,130, 131]. This technique is strongly species and genotype dependent [129], and there are only few reports on production of haploids in woody plants [130, 132]. There are two methods for *in vitro* production of androgenic haploids, *viz* anther culture and isolated pollen [microspore] culture.

3.1.1. Anther culture

Anther culture imparts an easy and one step protocol for haploid plant production [130, 132]. Although different species as well as different cultivars within a species show diverse requirements, there is no single specific condition or protocol for inducing *in vitro* androgenesis. The various steps of anther culture can be summarized as in figure 2.

For anther culture, appropriate size of flower buds was collected early in the morning. Stage of microspore development was determined by acetocarmine/DAPI/Shiff's reagent squash preparations. Anthers containing early-to-late uninucleate stage of microspores were cultured in the laboratory.
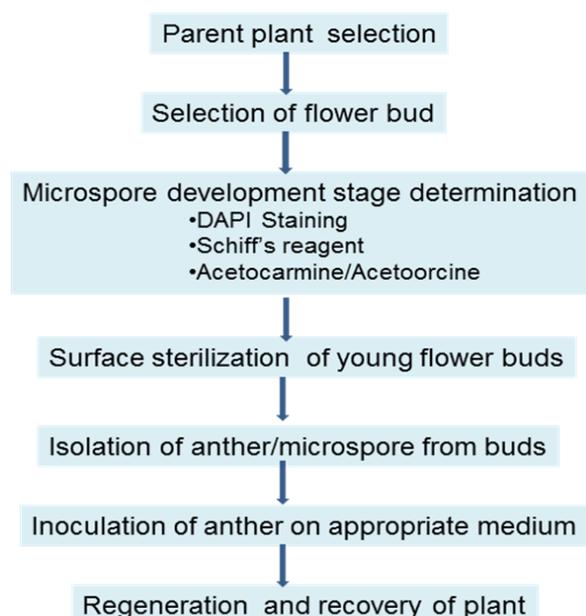

Figure 2: Common steps for haploid production via anther culture.

Picked flower buds from field are initially rinsed with sterile distilled water (SDW) several times followed by surface sterilization with surface sterilizing agent. Then the buds are washed more than three times with SDW. All steps are carried out in a laminar-air-flow system for avoiding condition. The surface sterilized buds are dissected under the stereo-microscope with the help of sterilized forceps and a dissecting needle in a laminar-air-flow system. Care should be taken to remove filament that is attached to the anthers to avoid *in vitro* callusing from the cut ends. The isolated anthers are inoculated in pre-poured medium containing Petri-dish (60mm diameter). Density of cultured anther kept around 1.5 anthers per ml of media. After inoculation the Petri-plates are sealed with parafilm and subjected to specific treatment conditions.

3.1.2. Pollen/Microspore culture

Anther culture is having a number of problems, major problems is the concomitant callusing of anther wall along with pollen. Moreover, the plants arising from an anther would constitute a heterogenous population. It has been also found in some species that asynchronous pollen development from anther culture, the older grains may suppress the androgenic capacity of younger grains by releasing toxic substances [3]. Callus formation in isolated pollen culture of *Brassica oleracea* and the hybrid *B. oleracea* X *B. alboglabra* was reported for the first time by Kameya and Hinata [133] and after that, successful pollen derived androgenic plants have been produced in several crop species. No universal protocol available for microspore embryogenesis of all species [134]. Höfer [135, 136] compared anther and isolated microspore culture and observed that the total number of regenerated plants was higher in case of anther culture, even though induction of embryo was higher in microspore cultures of some genotypes of apple. Microspore induction and regeneration were also slower in microspore cultures than anther cultures [134]. Regeneration differences occur among genotype within species from microspore culture.

Some important factors to be considered for optimizing the isolated microspore culture protocol are describing in Figure 2. In most of the cereals, pollen culture involves pre-culture of the anthers for a few days or co-culture of pollen with a nurse tissue. Treatment of pollen-derived embryos and pollen derived callus to recover complete plants is similar to that of anther culture. The nutritional requirements of isolated pollen in culture are more complex than

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those of cultured anthers. However, unlike the earlier belief, pollen culture is less tedious and time consuming than anther culture. The additional advantages of pollen culture over anther culture for haploid plant production are as follows:

1. A homogenous preparation of pollen at the developmental stage most suitable for androgenesis can be obtained by gradient centrifugation.
2. Isolated pollen can be modified genetically by mutagenesis or genetic engineering before culture and a new genotype can be selected at an early stage of development.
3. Pollen culture improves considerably the efficiency of androgenesis. In rapid cycling *Brasssica napus*, the culture of isolated pollen was 60 times more efficient than anther culture in terms of embryo production.
4. The exogenous treatments can be applied more effectively and their precise role in androgenesis studied as the unknown effect of the anther wall is eliminated.
5. The culture of isolated pollen provides an excellent system to study cellular and subcellular changes underlying the switch from gametophytic to sporophytic development and the induction of embryogenesis in isolated haploid single cells.

3.2. Haploid from female gametes

The use of the female gametophyte is an alternative way for production of haploids [137, , 29, 138, 139]. As described earlier *in vitro* gynogenesis is used as a alternate technique in species where anther/pollen culture is inaccessible or unsuccessful. The gynogenic plants may arise through direct embryogenesis or the gametic cells may form a callus followed by plant regeneration. Both ovary slice culture and ovule culture can be carried out simultaneously for achieving *in vitro* gynogenesis. Basic steps and factors involved in unfertilized ovary/ovule culture protocol are given in figure 3.

3.2.1. Ovary slice culture

For Ovary slice culture, unopened mature unfertilised flower buds, are collected early in the morning. The appropriate developmental stage of the embryo sac was determined by histological analysis, for histological analysis selected flower bud were fixed in fixed in FAA

[5:5:90 v/v/v Formaldehyde: Acetic acid: 70% Ethanol] for 48 hours and then stored in 70% alcohol.

The selected tea flower buds were surface sterilized followed by rinsing with sterile distilled water at least thrice. All the steps were performed inside laminar- air-flow cabinate. Flower bud were dissected and cut transverse section very carefully with help of scalpel under stereo-microscope. Sectioned ovary slice were cultured on appropriate medium. The sealed Petri-plates were subjected to various temperature and light treatments.

3.2.2. Ovule culture

The unfertilized ovary is surface sterilized with proper sterilizing agent and the ovules were taken and placed into culture. Excision of ovule, followed by culture on specific media may be either extremely easy to achieve, as in case of large-seeded plant species in which only a single ovule is present, or time-consuming and complicated, in small-seeded polyovulate plant species. Two kind of ovule support systems have been developed for ovule culture. First is filter paper support system and second is vermiculite support technique. In filter paper support technique involves culturing of the ovules on top of filter paper placed over liquid medium. In vermiculite support technique involves placing the ovules on a sterile vermiculite/liquid media mixture [vermiculite support]. In both techniques ovule always kept as micropylar side down. Unpollinated ovule culture has been used successfully for haploid production in sugar beets and onions. In case of *Nicotiana rustica* cv Rustica ovules with placenta were isolated from flower buds and were cultured on N_6 medium supplemented with growth regulators [140]. Since, there is usually one egg cell per ovule, ovule culture has much less potential than microspore culture [2].
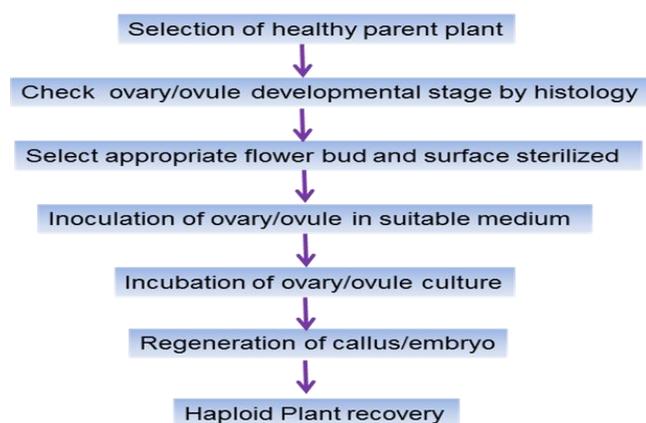
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Figure 3: Common steps for haploid production via ovary/ovule culture.

4.   FACTORS AFFECTING HAPLOID PRODUCTION

Since the first report of *in vitro* production of doubled haploid plants in the 1960s, there has been much progress in protocol development, efficiency, and application of the doubled haploidy method. There are several endogenous and exogenous factors which play important role in *in vitro* haploid production. The following pages contained the discussion on factors affecting haploid production and their possible mode of action in plants:

4.1. Genotype of the parent plant

The genotype of the donor plants are an important factor which has great influence on anther/ovary/ovule culture response and has been known since the early days of development of plants from pollen grains [141, 142, , 143, 29]. However, it is only in current times that the genetic factor affecting androgenesis as well as gynogenesis has been studied more intensively. Many plant species are quite recalcitrant in their *in vitro* response which is governed by specific genes on the chromosomes [144]. Specific regions of the chromosomes associated with the formation of embryo like structures [145]. It has been found that genotypic capability even exists between different cultivar types within the same plant species as has been reported in *Guizotia abyssinica* [34].

4.2.   Physiological status of the parent plant

In vitro androgenesis and *in vitro* gynogenesis much affected from the physiological condition of the donor plant *i.e* the environmental condition and age of the donor plant.

Physiological conditions of donor plant directly affect the induction frequency of pollen embryoids. A link between plant age and anther response has also been demonstrated by various workers. Generally the first flush of flowers yields more responsive anthers than those that are borne later. The frequency of androgenesis is usually higher in anthers harvested at the beginning of the flowering period and showed a gradual decline in relation to plant age [3].

4.3.   Developmental stage of explants material at the time of inoculation:

4.3.1. Stage of microspores

The pollen development stage is a complex factor that strongly affects the success of anther culture [1]. The stage of microspores at the time of inoculation is one of the most critical factors for induction of androgenesis. Detailed cytological studies conducted on poplar, rubber [128] and apple [49] have shown that androgenic callus and embryos were mainly induced through a deviation of the first pollen mitosis to produce two undifferentiated nuclei. Besides, affecting the overall response, the microspore stage at culture also has a direct effect on the nature of plants produced in anther culture [23]. In a vast majority of species where success has been achieved, anthers were cultured when microspores were at the uninucleate stage of microsporogenesis [130, 59, 146, 147].

4.3.2. Stage of the embryo sac:

The stage of embryo sac is an important determining factor for *in vitro* gynogenesis in various plant species. Unfortunately, there is not sufficient information available on this aspect. We cannot directly examine the embryo sac during culture without special treatments [any physical or chemical] that makes the further cultivation of ovaries impossible. So that the degree of development of the embryo sac is indirectly defined during the stage of development of pollen grain or with the help of histological preparations of ovules, that are at an identical stage the with cultivated ovules [29]. It has been published that the effect of ovule developmental stage on gynogenesis is profound as it harbours the embryo sac comprising of the egg cell. In several species have feature of protandry [male and female gametophytes do not mature simultaneously], maturation of anthers before carpels [e.g. onion, leek, sunflower, sugar beet, carrot etc.] and the opposite protogyny [e.g., pearl millet]. In niger

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there is no response of ovule culture which is collected two or three days before anthesis but before one day of anthesis about 5.0% and 13.3% embryogenesis were recorded [34]. Although a wide range of stage of embryo sac are responsive to *in vitro* gynogenic development, but, in most cases the later stages give better gynogenic response. It is quite different to anther culture in which mature pollen is not responsive to androgenesis. Late staged ovaries with mature embryo sacs gave good results in Barley and [25, 148, 149], others have published the success with ovaries ranging from uninucleate to mature embryo sacs [150, 151, 152].

4.3.   Culture media

4.4.1. Basic media

The constituents of the basal medium and combinations of growth regulators [with specific concentration] are also an important factor in eliciting successful androgenesis and gynogenesis. This is difficult to suggest one single culture medium with a particular growth regulator for all the systems. Regeneration of androgenic and gynogenic plant obtained either directly via embryogenesis or via callus formation followed by organogenesis from pollen/egg cell. In the later stages of in vitro development of plant the media constituents may differ according to culture conditions and requirement culture. In androgenesis most of the species required complete nutrient medium [mineral salts, vitamins and sucrose] with or without growth regulators. For anther culture the most common used basal media are N_6 [153], MS media with slight modifications [154], Nitsch and Nitsch [141] medium and B5 medium [155] etc. Initially in the 1950s used Nitsch medium for ovule and ovary culture, from 1970s Miller MS or N_6 media have been used. In *Gerbera*, MS basal medium seems better the Knop and Heller medium [156].

4.4.2. Growth regulators

Embryoids developed even in the basal medium of few plant species, namely *Datura innox* [157], *Nicotiana tabacum* [141], and *Hyoscyamus niger* [158, 159]. Several reports are available in which either one or other hormone has been found necessary for an androgenic response. The requirement of growth regulators and culture medium in terms of type and concentration may differ with each and every plant system. Usually, there is an agreement that the source and amount of total nitrogen as well

as combination of a cytokinin and an auxin is necessary for pollen embryogenesis and pollen callusing in several woody plants [3, 130, 128, 160]. It has been published in most plant of Solanaceae family required addition of an auxin to the induction medium is not a prerequisite for anther response but, the addition of auxins and cytokinins alone or in combination is crucial for microspore derived embryo production in majority of the plants and especially the recalcitrant ones [161]. The kind and concentration of supplemented auxins look to determine the pathway of microspore development [162] with 2,4-D inducing callus formation and IAA and NAA promoting direct embryogenesis [163, 164]. Giberrellins and abscissic acid have been rarely supplemented in the media.

Auxins are extensively used for induction of gynogenesis and their optimum concentrations have been reported to vary considerably from species to species [165]. Gynogenesis in Sunflower occurred only in presence of 2,4-D or NAA in the medium [166]. Combination of auxin and cytokinin was also reported to be useful for gynogenesis in allium species [167], mulberry [36]. On the other hand, in mulberry gynogenic haploids could only be produced on presence of cytokinin [BA or kinetin] suplimented medium [168]. These workers observed gynogenesis in *ab initio* ovary cultures of Mulberry.

4.4.3. Growth additives

The addition of other substances in the medium such as glutamine, casein, proline, biotin, inositol, coconut water, silver nitrate [ethylene antagonist] and polyvinylpyrrolidone has been reported [169, 170, 171]. The addition of glutamine [172, 173] and glutathione [141, 174] to the culture medium also enhances the embryogenic response.

Moreover, the exogenous addition of aliphatic polyamines [PAs] in the medium has been reported to increase the number of pollen derived embryos in potato [175], in some Indian wheat cultivars [176], in cucumber [177] and in clementine [178]. It is generally seems that PAs such as putrescine, cadaverine, spermidine and spermine are low molecular mass polycations which are involved in *in vitro* organogenesis and embryogenesis [179, 180]. In addition of undefined extracts, such as coconut milk [18, 19], potato extracts [181, 182, 174] and extract of anthers themselves [172] have also been used. The mode of action of these complex mixtures is unknown, but, they probably provide some substance[s] beneficial for cell divisions in pollen grains [183]. Similarly, the

use of additives in medium has also been reported in ovary culture. Maximum gynogenic response was observed in mulberry when excised ovaries from inflorescence segments were cultured on MS+BAP [8.5µM] +2, 4-D [4.5 µM] were transferred to MS +2, 4-D (4.5 µM) +glycine (6660 µM) + proline (1738 µM)[36].

4.4.4. Carbon source

Sucrose [disaccharide] has commonly used as the major carbon source in the culture medium. Sucrose concentration in induction medium has a major effect on osmosis and the development of embryos is apparently influenced by osmosis [184]. The effect of sucrose on anther culture has been investigated in a number of species. The necessity of sucrose for successful androgenesis was first demonstrated by Nitsch and Nitsch in 1969 for tobacco [141] and later by sunderland et al. [185] for *Datura innoxia* .Generally, sucrose is supplied at 2-3% concentration. However, increase in its concentration can lead to beneficial morphogenic potential [186, 147] by suppressing the proliferation of somatic tissues [187]. In case of neem higher concentration 12% of sucrose promotes induction of anther and lower concentration [3%] of sucrose faver further multiplication and regeneration [132]. All *Brassica* species require 12-13% sucrose for androgenesis in anther and pollen cultures. According to [188] high sucrose concentration favours better survival of pollen grains. The kind, type and concentration of carbohydrate used in the medium for inducing *in vitro* gynogenesis varies from species to species. High sucrose concentration [8-10%] in the culture medium has been shown to be helpful on some species like sweet potato [189] and onion [190]whereas in summer squash 9% sucrose was detrimental for production of any embryos [33].

4.4 Culture conditions

4.5.1. Effect of temperature treatment

One essential requirement common to practically all species when switching gametic cell development from the gametophytic to the sporophytic pathway is the application of an external stimulus. The embryogenic potential is usually triggered by stress pre-treatment. Different stress pre-treatments have been used, such as cold or heat shock high humidity, water stress, anaerobic treatment, centrifugation, sucrose and nitrogen starvation, ethanol, gamma radiation, microtubule disruptive agents,

electrostimulation, high medium pH, heavy metal pre-treatments are particularly popular approaches in anther and microspore culture [191, 192, 193]. In all the pre-treatment temperature treatment is considered to be the most effective to induce pollen embryogenesis development and it may applied to excised flower buds, whole inflorescences or excised anthers prior to culturing or after culturing in order to divert the gametophytic pathway to sporophytic developmental pathway. In *D. innoxia* and *H. niger*, cold pre-treatments [4^0C] have classically been administered to flower buds [194]. Heat shock [about 32^0C] is required to trigger microspore embryogenesis in *B. napus* [195], but other types of stress such as the application of gamma irradiation or colchicine are also successful. In *Cucumis melo* pollination of pistils with irradiated pollen was essential to obtain ovules capable of forming gynogenetic haploids [196]. Cold pre-treatment of spikes enhances the yield of embryos formed after anther culture in several cereals [191].A beneficial role of cold treatment on gynogenesis has been reported in some plant species whilst in others no significant effect on gynogenesis has been observed. Pretreating the capitula of sunflower at 4^0C for 24-48h before culture significantly increases the induction frequency [197]. Cai et al. [198] observed a promontory effect of cold treatment of the young panicles of rice at 7^0C for 1 day before ovary culture.

4.5.2. Effect of light

Dark incubation are seem to best for anther or microspore culture. Initial incubation of pollen culture of *Datura innoxia* [199], and *Annona squamosa* [160] in dark then transfer to diffuse light was found to be suitable. Isolated pollen cultures are more sensitive to light than anther cultures [200]. Similarly Chaturvedi et al. [130] and 132], observe maximum induction in anther culture in case of neem. In *Brassica juncea* [201] and *Hordeum vulgare [*202] species light is detrimental even for anther cultures.

4.6. Other miscellaneous factors

4.6.1. Anther wall factor:

There is sufficient evidence that the anther wall is an important factor for pollen embryo development [3]. Pelletier and Ilami [203] had demonstrate the concept of "wall factor", according to which the somatic tissues of anther play an important role in the induction of sporophytic divisions in pollen. Subsequently, nursing effect of whole anthers for androgenic

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development of pollen of same species and different species has been published [3]. So it is one of the important research subject in anther culture of woody plants is to avoid the over proliferation of callus from anther wall tissues and to achieve a high yield of pollen embyoids and pollen calli. In anther culture of most woody plants, both pollen calli or embryoids and somatic calli from anther wall tissues grew at the same time. The development of callus from somatic tissues of anther can be avoided by culture of isolated microspores. However, there are not much successful reports on microspore culture in woody plants [130].

4.6.2. Culture density

The culture density is a important factor in isolated anther and pollen culture [3]. Huang et al. [204] explained the effect of culture density on embryogenesis in pollen cultures of *Brassica napus*. In this study, the minimum density required for embryogenesis is 3000 pollen ml^{-1} of the culture medium but highest embryo yield was obtained at 10000 to 40000 pollen ml^{-1}. This high plating density is crucial only for the initial couple of days. Dilution of the density from 30000 to 40000 to 1000 pollen after 2 days of culture did not reduce the embryogenic frequency. Arnison et al. [205] also reported the effect of culture density in anther cultures of *B. oleracea*. The frequency of pollen embryogenesis was enhanced if the anther culture density was increased from 3 anthers per 4 ml to 12-24 anthers per ml of the medium. Cardy [206] reported that in *B. napus* the response was better when anthers were cultured at a density of 2 anthers per ml. Srivastava and Chaturvedi [132], in anther culture of neem for androgenesis culture density was 2anther ml^{-1}.

4.6.3. Effect of female flower position

In vitro gynogenesis is also affected by the position of female flower on plant stem. This factor affected induction of embryos from ovule cultures of *Cucurbita pepo* [33]. One of the possible explanations for enhancing responses of tissue culture could be attributed to indigenous hormonal level [207].

6. PLOIDY LEVEL DETERMINATION

Analysis of Ploidy level is an essential part of haploid and doubled haploid production programme .which can be carried out efficiently either by chromosome counting during mitotic and meiotic cell division or by flow cytometry.

Counting of mitotic chromosomes is easier and faster. Root tips are the most convenient source of mitotic cells. When roots are not available, young buds, leaves or callus can be used. Flow cytometry using DNA selective flourochromes has been considered to be a fast and reliable method for the measurement of nuclear DNA content.

Chromosome counting

The most common chromosome counting protocols use root tip, the chromosome number being established during mitotic cell division, although counting of mitotic chromosomes arrested in metaphase is generally easier and faster [208, 209]. When roots tip unavailable, young buds and leaves but also callus and cell suspensions, may be used instead. For chromosome preparation and staining applied may have to be modified according to the tissue source and, certainly, to the species studied. Three main basic operations for the handling of mitotic chromosomes are common to most species and include: [1] material collection and pre-treatment, [2] material fixation, and [3] preparation and staining of chromosomes.

The cytological procedures for visualizing chromosomes in woody plants may not be the same as in herbaceous species. The crucial step for chromosome counting involves proper chromosome squash preparation. It is very important to obtain sufficient well spread metaphase plates and proper physical separation of the chromosomes. However, method of chromosome staining applied for ploidy level analysis depends on plant species and chromosome size [210]. The most commonly used staining methods for chromosome counting are aceto-orcein or aceto-carmine [211], Feulgen [212] and DAPI [213] staining, which stain only the chromosomes while the cytoplasm remains clear.

Protocol for cytological analysis in Neem [130, 132]

Material fixation

- Healthy root-tips [1cm] from developed plantlets were collected at 10.30 am and rinsed with tap water.
- The root-tips were pre-treated with 0.02% 8-Hydroxyquinoline at 4ºC for 4 hours.
- Following which, the root-tips were fixed in a modified Carnoy's fluid containing absolute alcohol-chloroform-Glacial acetic acid-Methanol [7:3:1:1] for 48 hours.
- Finally the root-tips were preserved in 70% ethanol at 4ºC.

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Chromosome staining

- The root-tips from fixed roots were excised and placed in a mixture of 9 drops of 2% aceto-orcein and 1 drop of 1N HCl in a watch glass and warmed gently.
- After cooling, the individual root-tips were placed in a drop of aceto-orcein on a glass slide, covered with a cover slip, warmed gently and squashed.

Flow cytometric analysis

Recently a large number of articles on ploidy determination by flow cytometry approach for haplo-diploidization or chromosome doubling are available like Doležel et al. [214], Ochatt [215, 216], Ochatt et al. [217, 218], Sharma et al. [219]. Flow cytometry using DNA selective flourochromes has been considered to be a fast and reliable method for the measurement of nuclear DNA content [220, 221, 222]. Unluckily, its application in plant biology has been overdue, largely owing to the fact, that flow cytometry requires single cell suspension [223]. As plant cells usually compact tissues, methods had to be developed for the preparation of such suspensions. Even though flow cytometry is an extremely efficient technique with high degree of accuracy, the preparation of high quality plant samples for ploidy analysis remains a vital issue. For determination and interpretation of the haploid status of the regenerates, firstly tissue of known ploidy [as a reference] is analyzed followed by the unknown tissue whose ploidy is to be analyzed. Several methods exist to prepare plant materials for flow analysis of nuclear DNA content, dependent on the brand and model of the flow cytometer used, but all share various steps in common, the first of which is the isolation of nuclei from the plant tissues and roughly chop it in an appropriate buffer to release the nuclei as a suspension. In the several existing methods, the buffer will always contain the medium where nuclei will be suspended and the stain, and these will be used simultaneously or sequentially, using one of the various brands of ready-made buffers available in the commerce or preparing a ''home'' buffer [216]. Plant cells [as opposed to animal ones] are rarely spherical, which may cause error into the flow cytometry signals, and isolating nuclei [usually spherical and smaller in size than the width of the laser or UV beam] eliminates this concern.

Protocol for flow-cytometric analysis in Tea [Hazarika and Chaturvedi, unpublished data [9]

Preparation of nuclear sample:

- Well developed calli obtained after 2 months of culture initiation were used for ploidy analysis.
- Extraction of nuclei and the analysis were carried out via fine chopping of the calli placed in 1ml ice cold woody plant buffer.The woody plant buffer was prepared by mixing 0.2 M Tris HCl, 4mM $MgCl_2.6H_2O$, 2mM EDTA $Na_2.2H_2O$, 86mM NaCl, 10mM Sodium Metabisulfite, 1% Triton X-100 (v/v) and 2% PVP-10 (w/v) according to the protocol of Loureiro et al. 2007 with slight modifications. The pH of the buffer was adjusted to 7.5, filtered through 0.22 μm Polyvinylidene fluoride (PVDF) membrane filter and stored at 4ºC.
- The suspension containing the nuclei was mixed by pipetting up and down several times followed by filtering of the homogenate through a 30μm nylon mesh.
- The nuclear suspension was stained with Propidium iodide at a concentration of 50 μg/ml.
- Simultaneously RNAse at a concentration of 50 μg/ml was also added to the nuclei Ploidy analysis:
- The ploidy level was determined using a FACs Calibur cytometer (Becton-Dickinson, USA).
- All measurements were carried out in triplicate using fresh Tea leaves as an external standard. Using instrument gain [photomultiplier voltage and amplitude gain], the position of peak G_1 nuclei of the reference sample was established on channel 200 on a 1024 scale following which the instrument settings were kept constant and the unknown samples were run under the same parameters. The mean channel number of the unknown sample G_1 peak was determined and the DNA Ploidy was calculated according to the relationship:
  Sample Ploidy (integer) = Reference Ploidy X (mean position of the G_1 sample peak/ mean position of the G_1 reference peak)

6. APPLICATION OF HAPLOID PRODUCTION

Haploids have been exploited in a wide range of theoretical and practical aspects of plant biology

and genetics. It has numerous applications some of these will be summarized in the following.

7.1. Development of homozygous lines

Homozygous diploid plants are highly important for screening of high yielding lines. Obtaining homozygous diploid plants by conventional methods is difficult in perennials trees. From several decades to over a hundred years are required to obtain a pure line by means of successive inbreeding throughout many generations. The seed set by inbreeding in many trees is so low, usually only a few of ten thousandth or sometimes no seed can be obtained at all: therefore, it is impractical to obtain pure lines by inbreeding [128]. Doubling of haploids will give a doubled haploid line with 100% genetic homozygosity. This overcomes the slow process of continuous selfing over many generations to reach almost genetic homozygosity in a conventional breeding program. Moreover, conventional method of haploid production by inbreeding is impossible if the plant is strictly cross-pollinating in nature. On the other hand, homozygous diploid plants can be achieved in a single generation by diploidization of *in vitro* raised haploids by colchicines treatment.

7.2. Genetic studies

Reports show that recycling doubled haploid lines can quickly improve haploid frequency and fertility restoration [17, 224, 225, 226, 227]. Due to lack of right materials in research work, the progress in the study of genetics in trees is much slower than that in annual herbaceous plants. The genetics of a lot of vital economical characters has not been clearly illustrated. As a result, it is still unidentified whether the desirable characters of the parents will appear in their progenies. Only when crossing between different homozygous diploid plants is carried out can we gain a clear idea of dominance of genes controlling various characters and that these characters are monogenic or polygenic [128]. Furthermore, if we can use the haploid plants as samples of gamete, then we can obtain directly the recombination value between genes. Moreover, we can also use the haploid plants to study chromosome homology in genome or between genomes. Recycling of selected doubled haploid lines through recurrent selection or any other breeding scheme is a fast and powerful way to achieve genetic enrichment of the inbred carrying more favourable alleles for yield, pest resistance, stress tolerance and general agronomic traits [228, 229, 230, 231, 232, 233].

7.3. Induction of mutations

In general, majority of induced mutations are recessive and therefore are not expressed in diploid cells due to presence of dominant allele. Since, haploid plants have only one set of chromosomes, their dominant and recessive characters can be seen simultaneously on separate plants. Haploids may be considered as being mutants in their own right at a genomic level [13], and later on the genetic and phenotypic consequences of ploidy differences have been well described in maize [234]. It is extremely advantageous to provide a convenient system for the induction of mutations and selection of mutants with desirable traits in the absence of their dominant counterparts [3]. Haploids also applicable in isolation of mutations, which may be masked in a diploid [235, 236, 237]. In microspore culture, it is very effective to produce mutants by treating microspores with chemical mutagens at the uninucleate stage, and this will generate pure elite mutant inbred lines [238].

7.4. Obtaining new genotypes with alien chromosomes

The procedure of interspecific and intergeneric hybridization can be combined with anther culture techniques [239]. Thus, new genotypes with various reconstructed chromosome complements can be obtained after their chromosome doubling.

7.5. Genetic manipulation

Haploids are also applicable in detecting linkages associated with quantitative inherited characters and could be used in calculating recombination values between linked genes [240, 241, 242]. As microspore culture is a single cell system, it makes selection at the single cell level possible and furthermore offers novel prospects for genetic manipulation like mutagenesis and transformation etc. Direct gene transfer by microinjection technique offers the possibility of transgenic plant formation by using isolated pollen culture having high regeneration efficiency [2]

7.6. Cytogenetic research

Haploids are useful in several areas of cytogenetic research. These include production of aneuploids, determination of the nature of Ploidy, determination of basic chromosome number and evaluation of the origin of chromosomes.

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7.7. Somatic fusion

Another importance of production of haploids is in somatic fusion. Protoplast of haploid can be used to produce diploid fertile plant with unique combination of valuable character as cytoplasmic male sterility. Use of this will save labour and space compared with those required in classical technology [243].

8. CONCLUSION

There are several available methods for development of haploids and doubled haploids, of which *in vitro* culture technique is the most effective and widely used. This review states the genesis, recent advances and critical factors and their impact on *in vitro* haploid production in tree species. Here we summarized an extended and fascinating history of haploids in higher plants. In tree species particularly, where breeding is usually complicated and time consuming due to their long reproductive cycle, high degree of heterozygosity and complex reproductive biology, the potential of gamete biotechnology constitutes a great benefit in comparison with conventional breeding methods. The depth knowledge of the gametic embryogenesis process and the factors involves in morphogenic capability and development which will provide the effective deployment of gametic embryogenesis and haploid technology in the improvement of plant species. This has already reported in several annual crops such as Cruciferae, Gramineae and Solanaceae. The prospect of haploid and doubled haploid production as a powerful plant breeding tool and requires reliable tissue culture protocols, which can overcome several problems such as low frequencies of embryo induction, albinism, plant regeneration, plant survival and the genotype- and season-dependent response. Nevertheless, the increasing number of recent reports regarding haploid research in higher plant, shows the enormous interest in this useful breeding tool, and encourages us to look with optimism at its future applications in these vital crops.

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