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In vitro production of haploid plants

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Although several methods have been developed for producing haploid plants, the *in vitro* techniques are much more efficient than inter-specific hybridization or treatment with plant-growth regulators, temperature or irradiation. Androgenesis is the most universal of these techniques but ovule culture and the *bulbosum* method could complement or replace anther culture in those species or genotypes with less responsive male gametes. Genotype, environment, physiological status of the donor plant, and culture conditions and components all need to be taken into account when developing procedures for producing haploid and dihaploid plants. Suitable methods are already well established for a number of important crops. However, many problems, related to regeneration frequency, gametoclonal variation and albinism, are still unsolved. It is now clear that haploids and dihaploids form the ideal system for genetic manipulation in plants. Their key role in producing new theoretical and applied knowledge in plant science is an important aspect of our review.

Key words: Androgenesis, anther culture, dihaploidy, gynogenesis, haploidy, microspore.

Methods for producing plants from haploid gametes have been, are, and will be of great interest to plant geneticists and breeders. The first breakthroughs in this field were by Blakeslee *et al.* (1922) and the Bulgarian geneticist Kostoff (1941), who obtained haploid plants, by parthenogenesis in *Datura* and by androgenesis in *Nicotiana*, respectively. They proved that haploids occur spontaneously but at a very low rate (10^{-6} to 10^{-3}). The real breakthrough came with the studies of Guha & Maheshwary (1964, 1966) and Bourgin & Nitsch (1967), who obtained haploids from cultured anthers. Since then, haploid plants of more than 90 species belonging to 35 different genera have been produced (Heberle-Bors 1985; Genovesi 1990; Foroughi-Wehr & Wenzel 1993). The production of haploid plants of a number of crops is now a routine practice.

In this review we only present recent methodological achievements, since other aspects have been well covered by Heberle-Bors (1985), Foroughi-Wehr & Wenzel (1989, 1993) and Pickering & Devaux (1992). Progress in haploid and dihaploid production is still slow because of the low frequency of plant regeneration, genetic instability and high levels of albinism, especially in monocots.

Basic Trends

In Vitro *Gynogenesis*

Attempts to culture unfertilized ovules or ovaries have been made with many plant species but, in most cases, growth stopped at the callus stage. The method is, however, of particular importance in those crops where anther culture is unsuccessful or problematic. To date, successful gynogenesis to green plants has only been achieved with sugar beet, potato, cotton, ginkgo and onion (Van Geyt *et al.* 1987; Muren 1989; Castillo & Cistue 1993; Laurain *et al.* 1993; Slavova 1993; Diedericksen & Sacristan 1994; Hansen *et al.* 1994).

The procedure for gynogenesis is generally quite similar to the classical methods of culturing other plant tissues. Donor genotype is thought to play a decisive role (Zhu *et al.* 1981; Doctrinal *et al.* 1989; Keller 1990). As far as the developmental stage of the gynogenetic cells is concerned, it is accepted that a broad range of growth stages is suitable for growth induction (D'Halluin & Keimer 1986; Cappadocia *et al.* 1988). Regeneration rates are quite low; usually only one or a few embryos develop from an embryo sac.

The somatic origin of the gynogenetic formations is

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Table 1. Haploid production (+) in some important crops.

Crop	Procedure					
	Anther culture	Microspore culture	Ovule culture	*Bulbosum* method	*Hap* gene	Alien cytoplasm
Wheat	+	+	+	+	–	+
Maize	+	–	–	–	–	+
Rice	+	+	+	–	–	+
Barley	+	+	+	+	+	+
Oat	+	–	–	–	–	–
Triticale	+	–	–	–	–	–
Rape	+	+	–	–	–	–
Potato	+	–	–	–	–	–
Pepper	+	–	–	–	–	–
Tobacco	+	+	+	+	–	–

difficult to identify in advance. If haploid and diploid plants are produced simultaneously, the somatic origin of the non-haploids can only be identified in the offspring (Foroughi-Wehr & Wenzel 1993). If viable gynogenetic embryos originate from the egg cell, this can be confirmed by developmental studies during the first week of *in vitro* culture.

One way of inducing gynogenesis is pollination with gamma-irradiated or chemically-treated pollen. This method has met with limited success using potato (Foroughi-Wehr & Wenzel 1993), musk melon (Sauton & Dumas De Vaulix 1987), onion (Dore & Marie 1993), sugar beet (Hansen *et al.* 1994) and petunia (Raquin *et al.* 1989).

The *bulbosum* method is based on the chromosome elimination that occurs during early embryo development in some inter-specific crosses. The chromosomes of one parent are partially or completely lost; in nature such events lead to sterility but, if embryo rescue is performed, haploid plants can be obtained *in vitro*. The procedure was developed with *Hordeum vulgare* × *H. bulbosum* crosses (Kasha & Kao 1970; Jensen 1975; San Noeum 1976) and later applied to wild barley species (Jorgensen & Von Bothmer 1988) and rye (Fedak 1977). The method is now well-established. Various culture factors have been adopted and more compatible *H. bulbosum* genotypes have been selected (Devaux 1991). The dihaploid lines produced were not inferior to those from single-seed descent (Devaux 1987, 1988; Devaux *et al.* 1990a, b, 1993a, b). Nevertheless, the greater potential of androgenesis has limited the practical breeding value of the *bulbosum* method, including its use for crosses of wheat with *H. bulbosum* (Sitch *et al.* 1985). A similar mechanism of chromosome elimination is used in wheat crosses with maize, the results of which seem to be more promising (Laurie & Bennett 1987, 1988; Comeau *et al.* 1992).

Androgenesis

Genotype. It is accepted that plant genotype plays the predominant role in determining androgenic responses *in vitro*, with major differences between genotypes in their reaction under given culture conditions (Ochendou & Mc-Clenaghan 1993; Orshinsky & Sadasivaiah 1994). Culture procedure should be optimized for each genotype (Dunwell 1981) to decrease or even minimize this genotypic effect. The ability to culture any species can also be improved by selection and breeding for it, based on an increasing number of genetic data (Foroughi-Wehr & Wenzel 1993).

Genetic analysis of the heritability of *in vitro* culture response is still rare but considerable information exists for wheat (Bullock *et al.* 1982; Henry & De Buiser 1985; Tuvesson *et al.* 1989; Foroughi-Wehr & Zeller 1990), rape (Keller 1984), maize (Barloy *et al.* 1989), potato (Wenzel 1980) and barley (Dunwell *et al.* 1987; Knudsen *et al.* 1989; Larsen *et al.* 1991; Devaux *et al.* 1993a, b).

Donor-plant Growth Conditions. Early in the mid 1970s, the John Innes Institute pointed out the importance of the conditions under which the donor plants are grown (Dunwell & Perry 1973; Dunwell 1976). These early findings were confirmed for many plant species, including the recalcitrant cereals (Sun *et al.* 1993). For many crops, field-plants grown during the normal vegetative period were superior to greenhouse material (Pickering & Devaux 1992), probably because the latter is exposed to higher temperatures and more pest control (Foroughi-Wehr & Wenzel 1993).

Developmental Stage of the Microspores. It has been postulated that androgenic induction is only possible with immature anthers containing immature pollen (Heberle-Bors 1985). The early or mid-uninucleate stage of microspore development has been repeatedly confirmed as the most responsive stage of most species *in vitro* (Stoher & Zsuffa 1990; Hoekstra *et al.* 1992; Sharma *et al.* 1993). Embryos can be produced from older or younger microspores but at lower frequency (Summers *et al.* 1992; Atanassov & Djilianov 1995).

Pollen stage is easily verified by staining the anthers. The size of the flower bud containing the anthers gives

some indication of anther maturity but is affected by the bud's position in the inflorescence, genotype and the plant's age.

Procedures for culture induction have been studied in detail in various plants and reviewed. The microspore responses described for *Datura* are basically the same in other genera, with some modifications (Sunderland 1974).

Anther or microspore isolation can sometimes be avoided by culture of entire florets, tassels, spikelets or inflorescences (Pareddy & Greyson 1985; Kim & Raghavan 1988; Hou *et al.* 1993). Haploid plants are then obtained but at a lower rate than by anther or microspore culture.

Pretreatment of Anthers. Pretreatment of donor inflorescences, flowers and anthers should invariably be discussed in conjunction with the effect of genotype, the physiological status of the plant and the environment in which the donor plant is growing.

It was already accepted in the mid 1980s that the most effective approach to anther culture involved altering the temperature (Foroughi-Wehr & Wenzel, 1993; Osolnik, 1993). Cold pretreatments of various durations were recommended for most species (Pickering & Devaux 1992), although treatment of detached anthers was less effective than that of detached flowers (Sunderland & Wilson 1979). In some species (*Brassica campestris, B. napus* and *Capsicum annum*), heat pretreatments (at 30 to 45°C) of various duration give better induction (Keller & Armstrong 1978, 1979; Dumas De Vaulix *et al.* 1981). Cold treatment kills weak or non-viable anthers and microspores while arresting many of the microspores in the first mitosis, as starch production is blocked. Microspore and tapetal development then become asynchronous, influencing the switch from the gametophytic to the sporophytic phase (Pickering & Devaux 1992; Foroughi-Wehr & Wenzel 1993).

The exact mechanisms which act on plantlet formation following pretreatment are still unknown. The shock provided by low or high temperatures probably establishes cellular conditions which are favourable to embryogenic development. The low specificity of the trigger mechanisms is confirmed by similar stimulatory effects of other chemical or physical treatments. They are, at the same time, strongly genotype-dependent: spraying with ethrel in wheat (Bennett & Hughes 1972), potato (Tiainen 1992a, b) and barley (Evans & Batty 1994), centrifuging in tobacco (Nitsch 1974), low doses of γ-irradiation in rye (Stolarz 1974) and alfalfa (Zagorska *et al.* 1984) and rinsing in $NiCl_2$ solution in maize (Sung *et al.* 1993).

There are obvious similarities between androgenesis and morphogenesis, especially somatic embryogenesis from other plant tissues. Various pretreatments enhance the embryogenic ability of microspores, although this is affected by the endogenous hormone content of the donor plant tissues. Parallels can be drawn with the hormone content of the explants or donor plants in somatic embryogenesis systems. In alfalfa (Ivanova *et al.* 1994), the ratio of indole-3-acetic acid (IAA) to abscisic acid (ABA) in the initial explants has a great effect on embryogenic capability. Increasing ABA concentrations assure embryo maturation (Brown *et al.* 1993; Ivanova *et al.* 1994). The importance of this development in obtaining somatic embryos is evident and widely accepted (Atanassov 1993). The high frequency of callus or embryoid formation in genotypes that respond well without any pretreatment (Powell 1988) or even the negative effect of low temperatures on wheat anther response (Marsolais *et al.* 1984) could serve as indirect confirmation of this hypothesis.

Culture Media. In formulating media for haploid production, trial and error still predominate (Foroughi-Wehr & Wenzel 1993). The principles postulated for various types of morphogenesis in other plant tissues are also valid for anther cultures. There is a continually expanding list of media suitable for a range of species but most media are specific for a few or even only one genotype.

At the moment the most commonly used salt mixtures are those of Murashige & Skoog (1962) and Chu (1978) and an undefined potato extract mixture (Chuang *et al.* 1978). The Nitsch composition (Nitsch 1969) is most often used for tobacco. The salt composition of media has been found to have no effect on the frequency of the initial divisions, a moderate effect on plating efficiency, but a dramatic effect on embryogenesis and plant regeneration (Mordhorst & Lorz 1993).

The sugar used in the initiation and regeneration media is an important factor in numerous aspects of tissue culture (Navarro-Alvarez *et al.* 1994), high concentrations of sucrose (6% to 12%) being crucial for inducing androgenesis (Clapgham 1973; Wang *et al.* 1974; Sopory *et al.* 1978). The later stages grow better in or need lower sucrose concentrations (2% to 4%) (Sopory 1979).

There are now alternatives to sucrose as a main carbon source (Raquin 1983). Maltose greatly increased green-plant production in barley and wheat (Finnie *et al.* 1989; Hunter *et al.* 1989; Cai *et al.* 1992; Zhou *et al.* 1992; Hou *et al.* 1993; Meyza *et al.* 1993), probably it is hydrolysed more slowly than sucrose and prevents glucose accumulating at inhibitory concentrations (Roberts-Ochschlager *et al.* 1990). The replacement of sucrose as osmoticum has also been seen to give significant improvement in the overall response (Sorvari & Schieder 1987; Hoekstra *et al.* 1993). Liquid media are preferred for pollen embryo or callus induction. Kao (1981) reported a considerable increase in regeneration frequency when Ficoll was added to liquid medium and this effect has since been noted in several other studies (Foroughi-Wehr & Wenzel 1989; Zhou *et al.* 1992; Ghaemi *et al.* 1993).

Although agar was once the only gelling agent used to

produce solid media, more reproducible results have been obtained with Gelrite and various sea-plaque agaroses (Pickering & Devaux 1992). Recently, agar has also been replaced by starch (usually species-specifically), a solidifying agent with nutritional influences (Sorvari 1986; Foroughi-Wehr & Zeller 1990; Simonson & Baenziger 1992; Calleberg & Johanson 1993; Tiwari & Rahimbaev 1993).

Most experiments on the addition of other supplements to the media (hormones, amino acids, extracts of various origin, and vitamins) were carried out in the 1970s. Now efforts are being made to improve media by replacing some plant-growth regulators with others (Su *et al.* 1992; Ziauddin *et al.* 1992; Ball *et al.* 1993; Tiainen 1993) and adding amino acids (Trottier *et al.* 1993).

No single anther-culture medium can be recommended for general use. The concentration and composition of the sugars and the type of gelling agents used have the greatest influence on medium efficiency (Foroughi-Wehr & Wenzel 1993).

Culture Conditions. The ideal physical characteristics of a culture are species- and even genotype-specific. They particularly affect the final abortion rate and regeneration (Heberle-Bors 1985; Islam *et al.* 1992; He *et al.* 1993; Wolyn & Feng 1993).

Anthers are usually cultured in the dark at 20 to 30°C but regenerated plantlets are transferred to the light with a 16 h photoperiod and an intensity of 3000 to 10,000 lux. Optimum intensity is positively correlated with culture temperature (Foroughi-Wehr & Wenzel 1993). For field-grown material the optimal temperature for anther culture is generally 2°C higher than for greenhouse-grown material (Ouyang *et al.* 1987). The orientation of the anthers on the medium with respect to the loculi has a considerable effect in some species (Sopory & Maheshwari 1976; Hunter 1985; Shannon *et al.* 1985; Tsay *et al.* 1986).

Microspore Culture. Culture of isolated microspores has generally developed more slowly as a method than anther culture but the embryo production achieved is often much higher (Siebel & Pauls 1989). Field performances of plants produced by each method are similar.

There are two general ways to initiate the cultures: shedding of microspores into the liquid medium or their mechanical isolation from the anthers. Shedding is relatively simple and avoids damage to the pollen (Sunderland & Xu 1982) and the elimination of diploid anther tissue and of the competitive interactions within the anther makes it more suitable than mechanical isolation for gene transfer and selection *in vitro* (Foroughi-Wehr & Wenzel 1993). Faster and quantitatively greater embryo formation can also be achieved, without any interference from somatic cells derived from anther walls (Ziauddin *et al.* 1989; 1990). Studies on the cellular and molecular mechanisms controlling the switch from gametophytic to sporophytic development can be easily performed (Custers *et al.* 1994). The absence of diploid tissue is not always obligatory; conditioning of the liquid medium with anthers or ovules of some species (e.g. barley) was even found to improve culture effectiveness (Kohler & Wenzel 1985; Datta & Wenzel 1988).

Numerous methods for the mechanical isolation of microspore have been developed (Pickering & Devaux 1992). A cold-spike pretreatment assisted mechanical segregation because the resultant tapetal degeneration facilitated the squeezing of the microspores (Sunderland *et al.* 1984; Mollers *et al.* 1994).

Microspore culture can not yet compete with anther culture for dihaploid production, but might be used in studies on gametophytic to sporophytic transition in pollen, for transformation and for *in vitro* selection systems (Kasha 1989; Tuvesson & Ohland 1992).

Problems Still not Solved

Frequency of Haploid Production

After intensive studies with crops such as tobacco, potatoes and rape, the influence of genetic background on haploid frequency was decreased by improving the *in vitro* culture conditions. Relatively little attention has been paid to the effects of the physiological status of the explant and the endogenous hormone content on the androgenic response. An effective and economical procedure for haploid production can only be established following biochemical and molecular studies, especially with the 'recalcitrant' legume and tree species.

Dihaploidization and Genetic Stability

The plants produced by haploid procedures often have a level of ploidy above n or $2n$. Methods for dihaploid production are now more or less defined for most species (Foroughi-Wehr & Wenzel 1993; Iqbal *et al.* 1994). Use of such methods leads to a number of qualitative and/or quantitative changes apart from the required cytological ones (Guiderdoni & Chair 1992). It is obvious that haploid and dihaploid individuals can only be fully exploited in genetic and breeding studies when gametoclonal variation (due to residual heterozygosity, gene mutation, gene amplification, and lethality) can be controlled.

Albino Production

The production of albino plants remains a major obstacle to the use of haploidization in cereals. There are only speculations about the genetic basis of albinism in regenerated plants (Tuvesson *et al.* 1989; Larsen *et al.* 1991; Tiainen 1992b; Guiderdoni *et al.* 1992). Following the report by Day & Ellis (1985) on deletions in barley plastid DNA, Andersen, S.B. & Forster, B.P. (unpublished work) recently

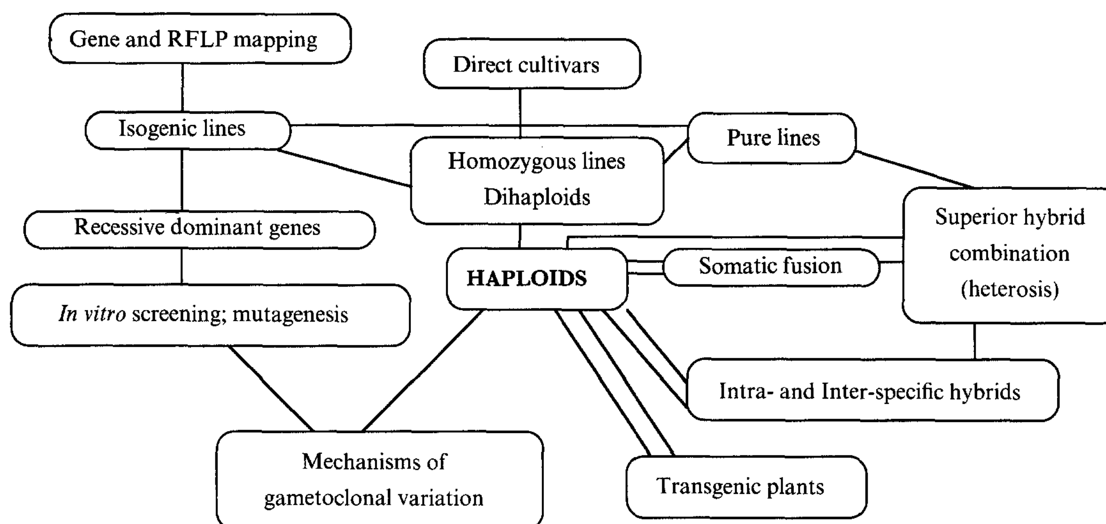

Figure 1. Applications of haploids and doubled haploids in fundamental and applied research.

began studies on the genetics behind albinism in wheat, using random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP).

Applications in Plant Breeding and Genetics

Haploid technology is among the most important trends in plant tissue culture, with both theoretical and practical value (Figure 1). It will continue to play a crucial role in facilitating the development and application of genetic engineering techniques, particularly within plant molecular biology.

Plant Breeding

In many crops (including tobacco, rice, barley, wheat, maize, rape, sugar beet and potato), haploid technology has already produced promising results. Several cultivars and valuable breeding lines have been released as doubled haploids and there is no reason to doubt that many more will be bred. Bulgaria is one of the countries which has already established high-yielding cultivars using this technique: the tobacco 'Biopreslavna' (Dimitrova *et al.* 1988) and the rice 'Mariana' (Boyadjiev *et al.* 1990; Boyadjiev 1991). The most successful results have been obtained in China, Canada, France, Germany and Denmark. The main advantage of the haploid and dihaploid lines is their complete homozygosity. This makes phenotypic selection for qualitative and, particularly, quantitative characters much easier and effective. In many tetraploid species, haploidization is used to produce dihaploids. Experiments are in progress to determine whether these dihaploids are of commercial interest. In species and genotypes where the regeneration of haploid plants is now routine, the questions remain whether and how dihaploids can be used more effectively in the F_1 or subsequent generations or if the haploid step would be better combined with other breeding techniques. For complex breeding characters, a combination of recurrent selection, especially after remote hybridization, and haploid selection is required. In such cases, the negative pleiotropic effect of some genes on agronomical performance allows an effective selection of polygenic traits in the first generation.

Genetics

Linkage Analysis. Haploids are important in detecting linkages associated with quantitative inherited characters and could be used in calculating recombination values between linked genes (Islam *et al.* 1992; Ekiz & Konzak 1994; Lashermes *et al.* 1994).

In Vitro Screening and Mutagenesis. Induced haploidy creates an opportunity to select for dominant or/and recessive traits at the haploid level. *In vitro* selection or/and mutagenesis can be applied. For example, after mutagenic treatment, recessive mutations are likely to be expressed in haploid regenerants if the traits are monogenic. Selective agents, such as herbicides, phytotoxins (pathogens) and heavy metals, can be used at different points in the haploid technology, to evaluate the efficiency of the screening system (Witherspoon *et al.* 1991; Henderson & Pauls 1992; Nichols & Rufty 1992; Shtereva *et al.* 1993).

Somatic Fusion. Haploid protoplasts can be used directly to produce diploid fertile plants with unique combinations of valuable traits as cytoplasmic male sterility. The saving of labour and space could be considerable compared with those needed by classical technology.

Gene Transfer. Some fundamental aspects of gene transfer, such as copy number effect, position effect, regulation

effect (methylation silencing etc) can be studied comparatively at haploid and dihaploid levels and in homo- and heterozygous dihaploids (Dupuis & Pace 1993; Zhou *et al.* 1993; Li *et al.* 1994).

Restriction Fragment Length Polymorphism. There is considerable interest in constructing a genome map, based on molecular markers and RFLP analysis. The location of polygenes controlling quantitative characters, such as yield, has been difficult, but could now be facilitated in many species by obtaining doubled haploids from crosses between parents with contrasting marker genes (Rivard *et al.* 1989; Devaux *et al.* 1993a).

Conclusions

The technologies for haploid and dihaploid production *in vitro* are now widely used to produce new cultivars and for genetic analysis of and molecular biological research on many important agricultural crops. Many problems, however, remain and these limit extensive application of the technologies in modern breeding programmes. During the next decade, a greater understanding of the mechanisms that govern the *in vitro* response of the plants is expected to be achieved, mainly through molecular biology (RFLP, RAPD, PCR etc). This will allow extensive application of the haploid procedure in crop improvement.

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