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Factors Affecting *In Vitro* Androgenesis in Cereals

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ABSTRACT

In vitro androgenesis is one of the most efficient methods to obtain haploid plants. Obtaining dihaploids of high frequency is of great importance for wheat breeding since in this way the possibility to create homozygous lines within one generation is provided, thus shortening the breeding process. In this review we consider and discuss many articles whose results show that wheat's response to anther culture is determined by genetic and environmental factors as well as by the interaction between them. The present state-of-the-art of the studies on the genetic control of *in vitro* response of wheat as well as on the prospects of haploid/doubled haploid induction in this important crop and its practical use are presented. The genetic factors affecting different stages of callus induction and organogenesis in anther culture are discussed on the basis of our and other authors' investigations. The dominant role of the genotype on *in vitro* processes and the influence of the cytoplasm are demonstrated. The interactions between the genotype-environment and that between the nucleus-cytoplasm are analyzed. A number of environmental factors influencing the response of anthers to *in vitro* cultivation are discussed. These include the growth condition of the anther donor plants, the developmental stage of the microspores, the culture conditions, and the medium composition. In spite of intense research in this field, many issues remain insufficiently clarified, and therefore need further investigations.

Keywords: anther culture, cytoplasm, genetic control, genotype, regeneration, wheat

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INTRODUCTION

Haploid and doubled haploid plants are useful tools for the genetics and breeding of staple crop plants such as wheat. Anther culture is one of the most efficient methods for the production of doubled haploids in cultivated crops. Haploids have considerable potential as breeding material in crop improvement programs. It is of great importance to obtain dihaploids of high frequency as in this way the possibility to create homozygous lines within one generation is provided (Devaux 1988). However, the insufficient androgenetic capacity of many genotypes is still a problem that restricts the use of this method in wheat breeding (Andersen *et al.* 1987; Henry and de Buyzer 1990). The problem is of both a technical and genetic nature.

According to Szakacs *et al.* (1989) the total production of wheat haploids via anther culture depends at least on three different and independent factors: The rate of embryo induction, the embryo regeneration ability and the green/albino plant ratio. The ability to regenerate green plants is the most crucial criterion which determines whether androgenesis can be utilized for a given wheat genotype in the breed-

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ing practice. The androgenic capacity, i.e. the number of responding anthers and callus yield, may also be used as a reliable trait indicating the efficiency of the anther culture techniques in wheat breeding (Sesek *et al.* 1988, 1992; Orlov *et al.* 1993; Sesek and Dencic 1996). The mechanism which prompts a change in the developmental pathway of microspores from the gametophytic to the sporophytic stage is still unknown. Induction of macrostructures (calluses and embryoids) commonly occurred three weeks after incubation of anthers as was reported by Armstrong *et al.* (1987), but this period of time can be influenced by the genotype and the culture conditions (Ouyang *et al.* 1983). In culture, microspores undergo various modes of androgenesis which lead to the formation of haploids either directly by embryogenesis, or indirectly via callus formation. Armstrong *et al.* (1987) characterized two regeneration systems in wheat anther culture: Embryogenesis and organogenesis. In the first system bipolar structures, such as zygotic embryos, are formed which germinate under appropriate conditions and begin to give rise to a whole plant. In the second system on regeneration medium the calli give rise to numerous shoots and thin roots. Formation of leaves and leaf buds are also observed. A big problem related to effectiveness of anther culture in cereals is the incapacity of obtaining a great number of albino regenerants. This is due to genetic reasons as well as to the method of anther cultivation.

Wheat response to anther culture is determined by genetic and environmental factors as well as by the interaction between them (Fadel and Wenzel 1990). In this article, these groups of factors will be discussed.

INFLUENCE OF GENETIC FACTORS

Genetic analysis of the *in vitro* response of wheat anther culture

Plant genotype plays an important role in the determination of the androgenetic response (Henry and de Buyzer 1985; Szakacs *et al.* 1989; Orshinsky and Sadasivaiah 1994; Balatero *et al.* 1995; Beltchev 2002). The great impact that the genotype-effect exerts on the response to *in vitro* cultivation is a major feature of wheat anther cultivation. Embryo formation and the percentage of green plant regeneration in wheat anther culture are strongly affected by the genotype of the donor material, while regeneration of the embryos into plants is less dependent on the genetic differences (Andersen *et al.* 1987; Tuvesson *et al.* 1989). Genotypic differences have been reported to account for 32-85.6% of the variation in green plants yield (Andersen *et al.* 1987; Tuvesson *et al.* 1989; Zhou and Konzak 1992; Masojc *et al.* 1993; Stober and Hess 1997; Maschii *et al.* 1998; Holme *et al.* 1999; Tuvesson *et al.* 2000). Agache *et al.* (1989) and Ziegler *et al.* (1990) found predominantly albino regenerants in certain cultivars. Ouyang *et al.* (1987) obtained an average of 44% green plants and 56% albino plants on three spring and one semi-winter wheat (*T. aestivum*) cultivars. Foroughi-Wehr and Zeller (1990) investigated the *in vitro* microspore androgenetic reaction of 25 commercial German spring (including four *T. durum*) and 50 winter wheat cultivars and they found significant genotypic differences. Anther culture response of 44 spring bread wheat (*T. aestivum*) cultivars, lines and F_1 crosses have been investigated by Ekiz and Konzak (1994b). According to these authors there were significant genotypic differences especially in callus induction and green plant production compared to plant regeneration. In a genetically wide material of winter wheat including 215 cultivars, Andersen *et al.* (1987) produced green plants in 93 genotypes with an average number of 1.3/100 plated anthers. In a study by Loschenberger and Heberle-Bors (1992), from an average of 30 hexaploid wheat cultivars, 73% of the regenerated plants were green. These significant differences in anther culture response of the wheat genotypes proved once more that the success of the anther culture method is strictly dependent on genotype.

Androgenetic parameters are polygenically determined and imply nuclear (Dunwell *et al.* 1987; Tuvesson *et al.* 1989; Ghaemi *et al.* 1994) or both nuclear and cytoplasmic effects (Powell 1988b; Sagi and Barnabás 1989; Ekiz and Konzak 1991, 1994a; Ponitka and Jarzina 1996). The utilization of aneuploids, addition, and substitution lines in wheat and rye allowed for the identification of chromosomes or chromosomal regions involved in the different stages of *in vitro* androgenesis (Zhang and Li 1984; Henry and de Buyser 1985; Lazar *et al* 1987; Szkacs *et al.* 1988; Agache *et al.* 1989; Foroughi-Wehr and Zeller 1990; de Buyser *et al.* 1992; Martinez *et al.* 1994; Dobrovolskaya *et al.* 2003). According to Shimada and Makino (1975), a genetic factor inhibiting callus induction is located on the β-arm of the 4A chromosome. Tuvesson *et al.* (1989) suggest that 1-2 dominant genes are involved in controlling regeneration ability. Zhang and Li (1984) showed by monosome analysis that several chromosomes, for instance 2A and 2D, carry main genes while chromosomes 5A, 5B, 4A and 2B carry genes with minor influence that inhibit the frequency of embryo production. Using aneuploid chromosome- substituted and translocation lines, de Buyser *et al.* (1992) found that genes located on chromosome 1D and $5B^L$ chromosome arms in 'Chinese Spring' increased embryo frequency, while a gene increasing albino plant frequency is located on the 5B chromosome. According to these authors few but highly efficient genes participate in the determination of anther culture in the *in vitro* response. Through reciprocal substitution analysis Ghaemi *et al.* (1995b) defined the effects on androgenesis of individual chromosomes and genes, as well as their interactions with the genetic background. For this, they used reciprocal substitutions for all chromosomes between the wheat cultivars 'Wichita' and 'Cheyenne'. They established that the A, B and D genomes were implicated in three different independently inherited traits: embryo induction, plant regeneration and frequency of green plant regeneration, with unequal participation. Embryogenesis was shown to be influenced by the three genomes, green plant regeneration – by genomes A and D, and albino and total (green and albino) plant regeneration – by genomes B and D. The authors identified specific chromosomes controlling androgenesis by using reciprocal substitution lines. The effects of chromosomes 1A, 7A, 1B, 5B, 1D and 2D on embryoids (E)/100 anthers were reciprocal, but 2A, 5A, 3B, 4D and 7D revealed non-reciprocal effects. The reciprocal differences in embryo yield indicated that alleles for an increase in embryo formation are present on 'Wichita' chromosomes (1A, 7A, 1B, 5B, 1D and 2D), and for the opposite effect on their homologues in 'Cheyenne'. As for non-reciprocal effects of chromosomes 2A, 5A, 3B, 4D and 7D, the authors concluded that changes in values of embryo production toward one donor genotype could be attributed to complementary interactions between genes on the substituted chromosome and those in the recipient background. In their experiments, green regeneration implicated all the chromosomes of the A and D genomes except 5D, but only chromosomes 2D and 7D had reciprocal effects. This indicated that genes increasing the production of green plant regeneration were present on 'Cheyenne' 2D and 7D, and those controlling the opposite effect – on their homologues in 'Wichita'. Chromosomes 1B and 4B, and all the D-genome chromosomes were implicated in total plant regeneration, with a reciprocal effect of chromosome 2D. Substitution lines of chromosomes 3D and 4D reduced total plant regeneration in both recipient cultivars. According to these authors, the genetic effect of these chromosomes on this trait is due to their negative interaction with the genetic background of the cultivar. There is information about the importance of some D-chromosomes for green plantlet formation (Galiba *et al.* 1986; Szakacs *et al.* 1989). Based on this information and the low rate of green plant regeneration in *T. durum* (Hadwiger and Heberle-Bors 1986), Loschenberger *et al.* (1993) investigated green plant regeneration in wheat species lacking the D-genome. Representatives of all tetraploid wheat carrying the AABB genome and diploid ancestors of common wheat, *Triticum monococcum* (AA)

and *Triticum tauschii* (DD) were included in their experiments. The formation of green plantlets clearly differentiated tetraploid from hexaploid wheat. Only 0.7% of the embryos of tetraploid wheat developed into green plants, while 21.7% of them developed into albino plants. None of the 10 regenerated plantlets of the DD-*T. tauschii* species were green. In hexaploid wheat cultivars, on the contrary, 73% of the regenerated plants were green (Loschenberger and Heberle-Bors 1992). More green plants than albinos were regenerated also in a genetically wide material of winter wheat (*T. aestivum*) including 215 cultivars (Andersen *et al.* 1987). Thus, it appears that the chromosomes of all three genomes of *T. aestivum* influence green plantlet regeneration (de Buyser *et al.* 1992; Agache *et al.* 1989; Szakacs *et al.* 1989). Possibly, there exists an advantage of the allohexaploid genome for green plantlet regeneration due to the interaction and compensation within and between genes on the chromosomes of the different genomes. Similar conclusions were made by Mentewab *et al.* (1997) in a study of different *Triticum* species with A, B, D and G genomes.

A significant increase in the responding number of anthers has been noted in the presence of 1B/1R recombination in the chromosome set (Henry and de Buyser 1985; Agache *et al.* 1989; Foroughi-Wehr and Zeller 1990). Muller *et al.* (1989) described 45 wheat cultivars and F_1 hybrids as also having a 1B/1R translocation chromosome and they observed a relatively high androgenetic response of these lines. However, there are cultivars that have a 1B/1R translocation which have low androgenetic ability. Genotypic differences were found among 1B/1R lines investigated by Muller *et al.* (1989). It was proposed that in addition to the 1B/1R translocation there are other genetic systems affecting microspore embryogenesis.

The influence of rye chromosomes on wheat *in vitro* morphogenetic processes and the interaction between rye and wheat genomes during the occurrence of these processes have been assayed by many authors. Nakamura and Keller (1982) pointed out a negative influence of 2R and 4R chromosomes of callus embryogenetic potential in substituted 2D/2R, 4D/4R and non-substituted triticale cultivars. Immonen (1992) reported a positive effect in some cases and a negative effect in others of the 2D/2R substitution. The author explained this by the interaction between rye and wheat genomes. Lazar *et al.* (1987) investigated wheat-rye addition lines and they stated that the 1R and 4R chromosomes carry genes that increase callus formation and regeneration in 'Chinese Spring' wheat culture. According to Henry and de Buyser (1985) the $1R^S$ arm carries gene(s) regulating regeneration potential. De Buyser *et al.* (1992) also reported that gene(s) involved in regeneration ability were located on the $1R^S$ chromosome arm. Later the location of "gametophytic" gene(s) able to stimulate plant regeneration from pollen embryos was confirmed by Henry *et al.* (1993). The chromosome 1R has been associated with *in vitro* androgenesis in a number of studies (Agache *et al.* 1989; Foroughi-Wehr and Zeller 1990; Henry *et al.* 1990; Martinez *et al.* 1994; Grosse *et al.* 1996; Dobrovskaya *et al.* 2003; Gonzalez *et al.* 2005). Gonzalez *et al.* (2005) established that four regions on chromosomes 1B, 1R, 4R and 7R were involved in the number of green plants/100 anthers. Grosse *et al.* (1996) reported the involvement of loci on the 3R chromosome in the androgenetic process in rye and mapped genes for anther culture ability in rye by molecular markers (Xpsr 902, Xpsr 116, Xpsr 598). Linkage analyses revealed a region on the long arm of chromosome 3R of line "DH5" to be associated with high induction rates and a region on the long arm of chromosome 1R with high regeneration rates. In line "DH3" there are hints that a region on the long arm of chromosome 5R is associated with high induction and albino rates whereas a region on the short arm of chromosome 6R correlates with high regeneration rate. Gonzalez *et al.* (2005) found that a region of chromosome 3R in triticale cv. 'Presto' was associated with green plant regeneration.

The relationship between the morphogenetic response of somatic calli and the androgenic response is very interesting. Agache *et al.* (1988, 1989) did not observe correlation between the behavior of the lines cultivated as anther culture and somatic tissue cultures, which indicates independent genetic control of both processes.

A number of researchers studied the influence of the heterogeneity of the donor material on *in vitro* androgenesis. There is much data concerning *in vitro* pollen embryogenesis in F_1 populations (Bullock *et al.* 1982; Deaton *et al.* 1987; Pauk *et al.* 1991). The regeneration ability of F_1 hybrids between hexaploid and tetraploid wheat was assessed by Masojc *et al.* (1993) and Ghaemi and Sarrafi (1994). Ghaemi and Sarrafi (1994) investigated the androgenetic capacity and green plant regeneration ability of 160 randomly selected heterozygous wheat (*T. aestivum*) genotypes. Pollen callus was obtained from 94% of the genotypes studied and the anthers of 68% of the genotypes tested produced green plants. El Maksoud and Bedo (1993) established that the ability of green plantlets to regenerate into embryoids obtained from F_1 hybrids was higher than the midparental values. Many researchers obtained similar results on heterosis in wheat anther cultures. Lazar *et al.* (1984b) reported the presence of heterosis over the midparental value. Ouyang (1986) found that 88% of F_1 hybrids showed heterosis for embryoid induction and 71% showed great heterosis for embryoid induction frequencies when the F_1 hybrids were compared to the better parent. It was also found that the ability of green plantlets to regenerate from F_1 hybrids was higher than that of midparental values. Therefore, the use of F_1 hybrid plants as anther donors is an effective means of increasing the yield of green haploid plants. Arzani and Darvey (2002) and Tyankova *et al.* (2003) also showed that heterozygous genotypes are particularly important for use in plant breeding. Gut *et al.* (2006) demonstrated that the haploidization of hybrids derived from the crossing of two or three forms may give most interesting results from a breeder's point of view.

Moieni and Sarrafi (1997, 1998) investigated the androgenetic response in two segregating populations (F_3 and F_4) and their parents. Their results indicated significant and positive differences between the F_3 generation and mid-parents for embryo production, green plant regeneration, and total plant regeneration. The difference between F_4 generation and mid-parents was positive and significant only for green and total plant regeneration. The best F_3 and F_4 plants produced 62.72 and 65.11 green plants per 100 anthers, respectively. As androgenetic traits are highly heritable, a rapid gain from selection of such F_3 and F_4 genotypes should be possible. It is evident that *in vitro* androgenesis depends on the heterogeneity of the donor material. The efficiency of dihaploid (DH) induction in cereal anther culture increases with the utilization of heterozygous plants as donors which makes the anther culture technique applicable to wheat breeding.

Anther culture ability is a heritable trait and can be transferred into agriculturally desirable material by crossing (Bullock *et al.* 1982; Foroughi-Wehr *et al.* 1982). Developments in DNA-based genetic marker systems have provided methods that can be used to identify quantitatively inherited traits into their qualitative trait loci (QTL) components via association with markers. These techniques have recently been used to identify chromosomal segments affecting anther culture response in maize (Cowen *et al.* 1992; Murigneux *et al.* 1994; Beaumont *et al.* 1995) and rice (He *et al.* 1998; Yamagishi *et al.* 1998; for review, see Bolibok and Trojanowska 2006). Recently, in wheat, the genes thought to be responsible for the genetic capacity for green plant regeneration in anther culture have been mapped in a population comprising 50 doubled haploid lines from a cross between two wheat varieties "Ciano" and "Walter" with widely different capacity for green plant regeneration (Torp *et al.* 2001). Bulked segregant analysis with AFLP markers and composite interval mapping detected four QTLs for green plant percentage on chromosomes $2A^L$ and $5B^L$. In a multiple regression analysis the four QTLs could explain a

total of 80% of the genotypic variation for green plant percentage. None of the chromosomal regions with QTLs for green plant percentage showed a significant influence on either embryo formation or regeneration frequencies from the anther culture. Zhang *et al.* (2003), using 57 dihaploid lines derived from a F_1 intervarietal hybridization in wheat, found 2 QTLs associated with the aptitude for green plant regeneration. Both QTLs were located on chromosome arm $5B^L$. Gonzalez *et al.* (2005) investigated QTLs involved in the androgenetic response in triticale. The regions involved in embryo induction found by these authors to reside in chromosomes 6B and 4R revealed the location of genes highly responsible for this trait (30.01%). Four regions on chromosomes 1B, 1R, 4R and 7R were involved in the number of green plantlets/100 anthers plated. The 4 QTLs for final yield of the process had a positive effect in triticale cv. 'Presto'. The joint effect of all four markers was highly significant and explained more than 46% of the variance. The authors explained the strong correlation between these traits by the proximity of the QTLs for embryo induction and for determining the total yield of the process. Their proximity, according to the authors, implies that they are jointly inherited. Another explanation assumed by the authors is the existence of a unique gene with a pleiotropic effect. Identification of distinct QTLs affecting the tendency for green/albino plant formation provides new tools for the study of these phenomena and for the identification of possible candidate genes.

Many researchers have studied the genetics of wheat anther culture. However, the results are not consistent with each other. The analysis of F_1 from parents of different androgenetic potential revealed intermediate inheritance of callus induction (Lazar *et al.* 1984b), while plant regeneration was often transgressively inherited (Agache *et al.* 1989). This implies that the *in vitro* frequency of androgenesis may be increased genetically. Schaeffer *et al.* (1982) also showed that *in vitro* androgenetic ability may be transferred from a cultivar of good androgenetic capacity to its F_1 hybrids. It has been demonstrated by means of reciprocal crosses that genes for green plant formation in wheat anther culture are mainly chromosomally inherited and show both additive and non-additive genetic effects (Charmet and Bernard 1984; Tuvesson *et al.* 1989; Zhou and Konzak 1992; Ermishina *et al.* 2004). Ekiz and Konzak (1994b) studied the anther culture of a large number of spring bread wheat genotypes and crosses between selected genotypes, and they found that additive genetic effects predominated for callus induction. In addition epistasis and heterosis were also observed in some crosses in agreement with the findings of Becraft and Tailor (1992). The authors established that some cultivars have dominant or masking genes suppressing callus induction which were functional in F_1 crosses as well. However, probably because of minor genes or gene interactions their functions were slightly altered positively in their crosses. On the other hand, additive epistasis and heterosis have been observed as genetic effects for plant regeneration and for the proportion of green plants, as well. In contrast to callus induction, genotypes with low proportion of green plants did not drastically reduce the proportion of green plants in their crosses. An insignificant correlation coefficient between anther culture components (callus induction, plant regeneration, and proportion of green plants) showed that these were under the control of different genetic mechanisms.

Apparently, the inheritance of anther culture components is complex. Depending on the genotypes used, different genetic mechanisms may be involved. However, it seems possible to improve the anther culture ability of genotypes through breeding so that the anther culture method can be used in practice more efficiently for haploid breeding and genetic studies.

Influence of the cytoplasm on *in vitro* androgenesis in wheat

Clarifying the genetic control of *in vitro* androgenesis is a complicated task because of the cytoplasmic genetic systems involved in the determination. According to Torp *et al.* (2001) nuclear genes affecting albino plant formation may exert their effect via interaction with events in plastid development. The cytoplasmic influence on the *in vitro* response of anther cultures has been studied by many authors. A controversial situation exists at present with regard to the involvement of cytoplasmic genes in pollen plant formation. In some experiments with reciprocal crosses, maternal effects were found (Picard *et al.* 1978; Bullock *et al.* 1982; Foroughi-Wehr *et al.* 1982; Charmet and Bernard 1984; Lazar *et al.* 1984b; Charmet *et al.* 1985). Similarly, in some cytoplasmic male sterile (CMS) lines, higher pollen plant yields were found as compared to those found in male fertile lines (Liang *et al.* 1987). In other CMS-lines, however, such higher pollen plant yields were not found (Picard and de Buyser 1973; Misoo and Mitsubayashi 1982; Misoo *et al.* 1984). Heberle-Bors and Odenbach (1985) showed that pollen embryogenesis does not depend upon the source of cytoplasm since highly embryogenic CMS-lines could be found with all cytoplasms included in the screening. On the other hand, not all CMS-lines with one particular cytoplasm were highly embryogenic, and this indicates that, in addition to cytoplasmic genes, other genetic (i.e. nuclear) factors must be involved. Picard *et al.* (1978) reported a great effect of *T. timopheevi* cytoplasm on the frequency of callus initiation in anther culture. The effect of cytoplasm was indicated by reciprocal differences in the individual components of the androgenic process in interspecific hybrids of triticale, barley and wheat (Charmet and Bernard 1984, Powell 1988b, Ekiz and Konzak 1994a). The genetic effects caused by the cytoplasm were not detected in other studies based on reciprocal differences (Dunwell *et al*. 1987; Agache *et al*. 1989). The apparent inconsistency is most likely due to the limited range of genotypes cultured in some studies as the expression of reciprocal differences is determined by the range of cytoplasmic and nuclear genetic variation among the parents. This has been demonstrated by anther culture experiments using alloplasmic lines, in which a *T. aestivum* nucleus was transferred to alien cytoplasms by substitution backcrosses. In those studies significant cytoplasmic effects and nucleus × cytoplasmic interactions were observed. Orlov *et al.* (1994, 1997) investigated a series of reciprocally substituted alloplasmic lines of wheat and their parental forms on parameters characterizing the plant's ability to induce morphogenetic processes in anther. Their results indicated a positive effect of the interspecific cytoplasm substitution on the expression of the investigated parameters (number of responding anthers, number of embryoids per 100 anthers and number of green and albino regenerants). Combining an alien genome and plasmone was shown to strongly modify their expression. Individual alloplasmic lines surpassed parental and corresponding "reciprocal" lines in their ability for embryogenesis induction and anther culture. A reliable approach suitable to reveal the involvement of the interaction between the cytoplasm and nuclear-cytoplasm in determining the parameters of "total number of embryoids" and "number of green regenerants" was shown. It is evident that cytoplasm substitution results in some cases in an increase in yield of embryoids and regenerants in wheat anther culture and may be used for improving the embryogenetic potential of tissue culture. Gordei *et al.* (1994) also pointed out that *in vitro* androgenesis depended on the alloplasmic state of the donor plants and on the possible use of the alloplasmic forms to enhance haploid induction in cereal anther culture. Ekiz and Konzak (1991a) tested two series of alloplasmic lines with *T. aestivum* cv 'Siete Cerros 66' or 'Penjamo 62' nuclei and they indicated that 5 cytoplasm types positively influenced all three components of anther culture response, or did not negatively affect any component while increasing one or

two of them. The yields of green plants per 100 anthers were 2 to 6 times higher than *T. aestivum* cv. 'Siete Cerros 66' or 'Penjamo 62'. Significant nucleus × cytoplasm interactions were observed between the nuclei and the alien cytoplasms. The results indicated a potential for use of alien cytoplasms to improve haploid production of wheat. Zhou and Konzak (1997) pointed out significant differences for callus induction, plant regeneration, and green plant percentages when nucleus of *T. aestivum* L. cv 'Selkirk' was transferred to ten alien cytoplasms by substitution backcrosses. In most cases, the alien cytoplasms decreased anther culture responses, but sometimes they were as good as or better than the *T. aestivum* cytoplasm. Arzani and Darvey (2002) demonstrated that due to the significant cytoplasmic differences in anther culture response the direction of a cross between genotypes was also an important factor in determining microspore development under *in vitro* conditions.

Embryogenesis in isolated wheat microspore culture

Little information concerning cytoplasmic effects on induction of embryogenesis in isolated wheat microspore culture is available. Orlov *et al.* (1999) tested different alloplasmic wheat lines for their ability to induce pollen embryogenesis in microspore culture. In experiments in which fertile alloplasmic lines were applied, a cytoplasmic effect was established. However, it is rather difficult to clarify the mechanisms of plasmone's participation in the genetic control of pollen embryogenesis characters. This participation may be of several types: to modify the influence on nuclear gene expression, their cytoplasmic control, joint nuclear-cytoplasmic control (Orlov and Palilova 1990). The mechanisms of cytoplasmic effects during pollen embryogenesis induction, according to Orlov *et al.* (1999), are supposed to be realized by the modification of the cytoskeleton in lines with alien cytoplasm. The authors concluded that cytoplasmic effects exist during early stages of pollen embryogenesis induction in wheat microspore culture. Microspore culture can be considered to be an efficient method for *in vitro* selection (Touraev *et al*. 1995) Preliminary studies with isolated microspore culture in wheat and *T. spelta* resulted in the production of embryos and in the regeneration of plants, but at a much lower percentage than with anther culture (Schmid 1990).

Recently, a number of sporophytically induced microspores and embryo-like structures (ELS) were obtained from isolated microspore culture of durum wheat (*Triticum turgidum* L. cv. 'Martondur 1') by Bakos *et al*. (2007). They tested various pretreatments involving spike treatment at 4°C for 2, 7 or 14 days, anther treatment in 0.4 M mannitol at 33°C for 3 days, and various combinations of these. The best results were achieved when starvation at high temperature was combined with no or short (2-day) cold treatment (212 and 203 ELS/100 anthers, respectively). However, the ELS failed to regenerate and only a few of them produced poorly developed albino shoots. Labbani *et al*. (2005) studied the effects of different pretreatments on the behavior of *in vitro* isolated microspores populations of durum wheat. They used two durum wheat varieties (*T. turgidum* subsp. *durum*) 'Cham 1' and 'Jennah Khetifa' (JK). The authors tested five different pretreatments: cold pretreatment during 3 days and during 5 weeks at 4°C, pretreatment using a 0.1 M mannitol solution at 4°C for 3 days, pretreatment combining first a cold phase (4°C) during five weeks, followed by mannitol (0.1 M) during 3 days at 4°C and the reverse one, i.e. in mannitol (0.1 M) during 3 days at 4°C, followed by a cold phase (4°C) during five weeks. Their results show that the microspore culture response varies within the cultivars and depends also on the pretreatment. The two durum cultivars showed a great difference in their embryogenic potentialities. Cv. 'Cham 1' had a poor response in embryo formation for all pretreatments. Cv. 'JK' showed a good embryogenesis for all pretreatments, especially when mannitol (0.1 M) was used for three days at 4°C. Under this condition, 0.71% of embryo production was obtained. Cold pretreatment for 5 weeks at 4°C was the best one for embryo production as well as for green plant regeneration. Labbani *et al*. (2007) obtained even better results by combining cold pretreatment for 7 days with 0.3 M mannitol solution. They observed that the novel pretreatment had a strong effect on the number of embryos produced and regenerated green plants. High green plant regeneration frequency was recorded. As an average 11.55 green plants were produced per 100,000 microspores (about the equivalent of six plants per spike).

Pauk *et al*. (2000) compared the effects of plant growth regulator (PGR)-free and PGR-supplemented media on triticale androgenesis. For this purpose, winter triticale cv. 'Presto' and four different F1 combinations were used as anther donor genotypes. Microspore donor tillers were cold pretreated at 4°C for about two weeks. The basic 190-2 medium (Zhuang and Jia 1983) was supplemented with 3 mM L-glutamine and the following growth regulator combinations: 1) 190-2 + 1.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/l kinetin; 2) 190-2 + 10 mg/l phenylacetic acid (PAA); 3) 190-2 without growth regulators. The results showed that the induction media had a strong effect on the number of embryo-like structures. The medium without growth regulators caused significantly higher level of embryogenesis compared to the two growth regulator-supplemented media. The highest number of ELS per culture (118) the authors recorded for "Presto × Monico" in the growth regulator-free induction medium. This culture medium induced 3 to 118 ELS per culture depending on the genotype. The two different growth regulator-supplemented media induced ELS at 5.8 to 20.7 per culture. The effect of medium was not significant on regeneration of albino plantlets and there was only a weak effect on the green plantlet production. The addition of 2,4-D and kinetin or PAA had no significant effect on any of the three parameters scored (number of ELS, green and albino plant regeneration).

In *T. aestivum*, first reports of isolated microspore-derived green plants were published by Meiza *et al*. (1993) and Tuvesson and Ohlunt (1993) almost at the same time. Different methods – treatment with inducer chemicals and ovary-conditioned medium – were developed to improve the efficiency of embryo production of isolated microspores (Zheng *et al*. 2001; Zheng *et al.* 2002). Importance of ovary co-culture in successful microspore culture of wheat was first published by Meiza *et al.* (1993). They found a positive effect of ovary co-cultivation on embryogenesis in float anther culture wheat. A significant positive effect of ovary co-culture was found also by Puolimatka *et al.* (1996), Indrianto *et al.* (2001) and Lantos *et al.* (2005, 2006). Ovary co-culture is necessary to production of a lot of embryoids, because ovaries extract some chemical substances which protect the developing structures (Letarte *et al.* 2006). Very important factors for successful culture are the osmotic pretreatment of donor anthers and optimal microspore stage. The late uni-nucleate and early bi-nucleate stages are the optimal for microspore culture (Reynolds 1997).

Highly efficient system for doubled haploid production in wheat via induced microspore embryogenesis has been developed by Liu *et al.* (2002). Over 50% of the total microspores in a spike routinely can be induced to become embryogenic by treatment with a formulation, including 2-hydroxynicotinic acid (2-HNA) at 33°C. Thousands of green plants originating from the microspores of a single wheat spike can be produced. The optimal concentration of 2-HNA in the formulation for treating microspores to induce embryogenesis and form mature embryoids was determined to be approximately 100 mg/l. The number of induced embryoids increased with the increase of the concentration of 2-HNA up to 100 mg/l, while the percentage of germinated green plants did not significantly differ between different concentrations of 2HNA. All eight genotypes studied responded to their method and formed green plants, but genotypic differences in response to 2-HNA, ability to form em-

bryoids and green plants were observed.

Although large populations of embryogenic microspores were obtained and cultured in nutrient media in the study of Liu *et al*. (2002), the majority of the developing embryoids ceased cell division in the process toward forming mature embryoids in media without the presence of live ovaries. This indicates that the female part of the wheat reproductive system plays an essential role for the reprogramming from gametophytic to sporophytic development. The authors found that extracts of ovaries were not active. Female nurse substances, only synthesized by live ovaries, were responsible for nursing the majority of embryogenic microspores to become mature embryoids. It was shown that there were no significant differences in the nurse function for androgenesis among the live ovaries of the different wheat genotypes tested. Even oath and barley ovaries were found to have similar nurse effects for androgenesis (Konzak *et al*. 2000). This finding indicates that universal mechanism, present in ovaries of any given wheat genotype, effectively provides nurse factors for androgenesis.

Pauls *et al*. (2006) reviewed the cellular and molecular changes in early-stage microspore cultures in several species (*Hordeum vulgare*, *Brassica napus*, etc.). Responding cells in those cultures have been found to enlarge, their nuclei – repositioned to their cell centers and their cytoplasm – filled with fragmented vacuoles. An early ultrastructural change that occurs in embryogenic microspores has been found to be the formation of a preprophase band of microtubules. The preprophase band marks the position of the new cell wall that develops between the equally sized daughter cells (Simmonds and Keller 1999). In contrast, the preprophase band is absent during normal pollen development (Scheres and Benfey 1999). According to Pauls *et al.* (2006) these observations indicate that the cytoskeleton plays a vital role in reshaping microspores committed to the embryogenic pathway. Various mechanisms for induction of embryogenesis in the cultured microspores have been proposed including novel mRNA synthesis (Touraev *et al.* 1997), protein phosphorylation (Garrido *et al*. 1993), and initiation of symmetrical cell division (Zaki and Dickinson 1991). Flow cytometry was used by Schulze and Pauls (1998, 2002) to track cellular changes in *B. napus* microspore cultures, as well as microarray analysis and real-time PCR to compare gene expressions in embryogenic and non-embryogenic cells. Pauls *et al*. (2006) discussed a model for embryogenic cell activation in plants that involves alkalization, Ca^{2+} signaling and changes in guanosine triphosphatase (GTPase) activity that lead to significant changes in gene expression. According to them, a shift from pollen formation to embryo formation in the microspores would be expected to trigger large shifts in gene expression. The authors grouped the variety of transcriptome changes that have been observed in induced microspore culture into three broad categories: 1) associated with adaptation to stress; 2) involved in a cessation of pollen development; 3) involved in the acquisition of embryogenic characteristics. They suggest that a number of the gene products, induced in these systems, are related to stress adaptation, as the common trigger for embryogenesis in a variety of microspore culture has been found to be stress of some kind. The authors concluded that an integrated set of changes in gene activity occurs in the microspores committed to form embryos, which indicated that these cells are active and performing new functions.

Microspore culture can be used for genetic transformation experiments, mutation studies, the investigation of mechanisms of plant differentiation and morphogenesis, the production of DH lines, etc. The use of DH populations simplifies breeding for recessive traits because homozygous recessive individuals occur at a greater frequency in these populations compared to conventional breeding populations (Pauls 1996). Culture of isolated microspores provides many advantages over anther culture. The isolation of microspores, as it is supposed by some authors (Touraev *et al*. 1996), might be used to overcome the genotypic limitations in anther culture. Besides, all embryoids formed in the culture are certain to be microspore derived and plants regenerated are either haploid or dihaploid because the only cells placed in the culture are microspores and there is no participation of the anther wall in pollen formation (Touraev *et al.* 1996). Microspore cultures have become model systems for studying the very early stages of embryogenesis. The fact that they are single-cell suspensions in liquid media makes continuous monitoring of the process possible and allows the system to be easily manipulated.

EFFECTS OF NON-GENETIC FACTORS

Besides genotype, a number of environmental factors have been reported to influence the response of wheat anthers to *in vitro* culture. These include the growth conditions of the anther donor plants (Wang and Chen 1980; Jones and Petolino 1987), the developmental stage of the microspores (Ouyang *et al.* 1973; He and Ouyang 1984; Hassawi and Liang 1990), pretreatment of anthers, the culture conditions (Jing *et al.* 1982), and the medium composition (Liang *et al*. 1987).

Effects of the donor plant's growing conditions

The effects of the donor plant's growing conditions (including seasonal variation and plant age) on pollen plant formation in anther cultures have been studied by a number of scientists (Picard and de Buyser 1973; Foroughi-Wehr *et al.* 1976; Keller and Stringam 1978; Sunderland 1978). The growing conditions of the donor plants are an important factor influencing the *in vitro* culture response. Plants growing in the field had a better androgenic reaction than those growing in the greenhouse (Ouyang *et al.* 1983). Andersen *et al.* (1988) confirmed this observation in some important Scandinavian varieties of winter wheat, but they obtained a great percentage (8.8%) of albino regenerants. Tuvesson *et al*. (2000) also showed that the regeneration of green plants is higher in material from the field. In the experiment of Orshinsky and Sadasivaiah (1997) with donor plants from three temperature regimes anthers from plants growing in higher day/night temperature (25/18°C) or anthers from plants transferred from low (15/12°C) to high (25/18°C) temperature gave more embryoids and green regenerants than anthers from plants that were grown at 15/12°C. Investigating embryo formation and plant regeneration from cultured anthers in 5 genotypes of wheat (*T. aestivum* L.), Jones and Petolino (1987) did not establish significant differences between the 3 sets of growth conditions studied (15°C-16/8h light/dark, 20°C-16/8h light/dark, and 20°C-12/12h light/dark). However, they observed significant genotype and genotype × environment interactions for embryo formation. Androgenic reaction is influenced by the season, as was pointed out by de Buyser and Henry (1979). Plants growing in spring had a better androgenic response than in the other seasons. This was not confirmed by Ziegler *et al.* (1990) who found that the rate of responding anthers, number of embryoids produced, and total plant regeneration indicated that season had no significant influence on anther reaction and subsequent regeneration.

Important factors governing the choice of donor plants are: photoperiod, temperature, supply of water or nutrients, application of growth regulators and pesticides. All of these factors are thought to play a role in the induction of androgenesis. According to Bajaj (1983) high light intensity before meiosis and low temperature, which lengthens the vegetative period, can enhance the induction of androgenesis. Stress such as water deficiency or some nutrient elements, for example nitrogen (KNO_3, 1150 mg/l), can also have an inductive influence (Feng and Ouyang 1988). In general, the physical state of the donor plants is important, but until now it has not been possible to clearly define single factors responsible for the success of anther culture.

The developmental stage of the microspores is a very important factor for anther callus induction. It was found that the middle mononuclear stage is optimal for the culti-

vation of anthers. According to Gang and Ouyang (1984) in hexaploid wheat the frequency of green plantlets was higher at the mid and late uninucleate stage than at the early uninucleate stage while that of albinos was the same in both stages.

Effect of anther pretreatment

Pretreatment of spikes or anthers is another factor that influences wheat anther culture. The microspores are physiologically/genetically determined to follow the gametophytic developmental pathway, but the mechanism which causes a change in this developmental pathway remains unexplained. In order for the pollen to change from gametophytic to sporophytic developmental pathway, a proper stress should be applied during pretreatment of spikes or anthers. The most frequent methods used are chilling cut-off spikes and immersing them or anthers in mannitol solution. Both techniques limit the intake of nutrients by microspores. In the case of chilling, the reason is the lack of synchronous development of the tapetum and microspores (Powell 1988a); with mannitol, the cause is the increased osmotic pressure in the solution in which anthers are immersed (Hoekstra *et al.* 1997). In barley Roberts-Oehschlager and Dunwell (1990) reported that a period of 4 days preincubation at 25°C on a 32 g/l mannitol-containing medium was superior to those pretreatments requiring incubations at 4°C. For induction of androgenesis to occur, it is sufficient to separate stems from the plant (Wilson *et al.* 1978) or to choose a medium with stress-inducing components, such as a suitable dose (e.g. 20 M) of 2,4-D (Hoekstra *et al.* 1997) or maltose, e.g. 0.7 M (Kruczkowska *et al.* 2005) as the source of carbohydrates in media inducing androgenesis in cereals. Due to the slow hydrolysis of maltose, microspores remain under starvation stress during the first 3-4 days of culture (Indrianto *et al.* 1999).

One of the key points to the developmental changes leading to androgenesis is starvation which seems to be essential for the shift of the developmental pathway of pollen from gametophytic to sporophytic (Wei *et al.* 1986). In many cereal species, cold treatment is necessary to get androgenic microspores in culture (Kao *et al.* 1991) and the function of cold treatment is perhaps starvation. However, the effects of cold pretreatment of spikes have not yet been ascertained and the available results are rather contradictory. Cold pretreatment is known to delay degeneration of microspores and accelerate nuclear division during the induction process (Raina 1997). Cold pretreatment has been extensively used in wheat anther culture and the best results were obtained when spikes were pretreated (before anther excision) at 4°C for 7 to 14 days (Huang 1990). There are several positive reports on the effectiveness of cold pretreatment of spikes in wheat anther culture. According to Heberle-Bors and Odenbach (1985), Datta and Wenzel (1987) and Benito-Moreno *et al.* (1988) cold pretreatment increases haploid or spontaneously doubled haploid formation in wheat. According to Lazar *et al.* (1990) cold pretreatment at 4°C for 14 days followed by culture in liquid medium increases the regeneration ability more than that at 10°C or 25°C for 7 days for the highly responsive genotype 'Chris' (31.2 vs. 7.9 and 0 per 100 anthers plated, respectively). A strong positive effect on spike cold pretreatment was also reported by Stober and Hess (1997) for a number of spring wheat cultivars. In the study of Ponitka and Slusarkiewicz-Jarzina (1996) the efficiency of androgenic embryos was the highest when spikes were incubated at 4°C for 6-9 days. On the other hand, cold pretreatment in general was not always necessary to obtain higher rates of anther culture response (Marsolais *et al.* 1984; Karimzadeh *et al.* 1995; Xinias *et al.* 2001). Furthermore, Karimzadeh *et al.* (1995) indicated that cold pretreatment usually has a negative effect on the androgenic ability of wheat genotypes and therefore it reduces embryo production. This negative effect indicates that for the genotypes studied cold pretreatment is not essential for androgenic induction. These results confirm the findings of Marsolais *et al.* (1984) and McGregor and McHughen (1990) in hexaploid wheat. Marsolais *et al.* (1984) found that cold pretreatment had a greater inhibitory effect on the proportion of responding anthers, than on the number of embryoids or calli produced. According to McGregor and McHughen (1990), a cold pretreatment prior to cultivation at 30°C caused a significant decrease in callus formation, regeneration of green plants and albino plants as compared to non-cold-pretreated anthers (for cv. 'Chris': 4.5%, 1%, 0% vs. 9.5%, 3%, 0.5%, respectively). The results of Karimzadeh *et al.* (1995) are in agreement also with the ones of Ghaemi *et al.* (1995a) who reported that cold pretreatment of spikes (7 days at 4 ± 1°C) usually reduces embryo yield in tetraploid wheat. A strong inhibiting effect of cold pretreatment on *in vitro* androgenesis in hexaploid wheat was also observed by Mentewab and Sarrafi (1998) and Simonson *et al.* (1997). Ohnoutkova *et al.* (2000) demonstrated that cold pretreatment is not an indispensable factor for changing the developmental pathway of microspores. Thus, the effect of cold pretreatment of bread wheat anther culture is questionable. Perhaps, cold pretreatment is genotype dependent and there exists a strong interaction between genotype × medium × cold pretreatment (Ghaemi *et al.* 1995a). According to Powell (1988a), Jähne and Lörz (1995), Lezin *et al.* (1996) and Ohnoutkova *et al.* (2000) the optimal length of cold pretreatment depends on the genotype, on the growth conditions as well as on the physiological status of donor plants. Different pretreatment procedures were used by individual authors. Chilling spikes at 4°C may be effective with different treatment periods. The most frequent treatment period for chilling spikes at 4°C is 28 days. Ohnoutkova *et al.* (2000) obtained positive results with short term chilling which lasted 1, 7 or 14 days. Lezin *et al.* (1996) pointed out that a 14-day chilling period had the best effect on embryo frequency as well as on the frequency of green plants. Devaux *et al*. (1993) showed a significant advantage of a longer chilling period (28 days vs. 14 days). Huang and Sunderland (1982) recommended a period of 21-35 days. In the experiments of Ritalla *et al.* (2000) after more than 4 weeks of cold treatment, the regeneration capacity decreased dramatically, but Hou *et al.* (1993) demonstrated that prolongation of the chilling period up to 42 days did not exert any negative influence. These conflicting results might be explained by the fact that the optimal length of the cold treatment depends presumably on the genotype (Powell 1988a), as well as on growth conditions and the physiological status of donor plants (Jähne and Lörz 1995; Lezin *et al.* 1996; Ohnoutkova *et al.* 2000).

In the method with mannitol, isolated anthers are immersed in 1.3 M mannitol solution at 25°C for 4 days. Some authors solidified the mannitol solution with agarose. Manninen (1997) obtained good results of androgenesis in some cultivars with 0.175 M mannitol solution. Cistue *et al.* (1994) showed that there were no significant differences between liquid and solid media with mannitol. They recommended increasing mannitol concentration up to 0.7 M for responsive cultivars. For recalcitrant ones, Castillo *et al.* (2000) suggested mannitol increasing up to 1.5 M. Starvation usually lasts 3 or 4 days, while 2 and 5 days are less favorable (Hoekstra *et al.* 1992).

Several authors compared the efficiency of the two pretreatment methods (cold and mannitol). Ziauddin *et al.* (1992) achieved similar results for both methods. According to Hou *et al.* (1993) the two pretreatments induce a similar number of embryos, but mannitol causes poor plant development. In the study of Castillo *et al.* (2000) pretreatment of anthers with a high concentration of mannitol produced a 3-10–fold increase in the number of green plants compared to anther chilling. Li *et al.* (1995) established that mannitol treatment, compared to chilling, significantly increased the survival rate of pollen in culture, accelerated its development, and led to higher percentage of DH plants. In the experiments of Kruczkowska *et al.* (2002) pollen viability in barley decreased after pretreatment, and the reduction was greater after treatment with mannitol (0.3 M) than after

chilling. In their experiment, chilling significantly favored the formation of embryos/calli in all the examined cultivars, but the ability of the embryos/calli to develop into plants in general and green plants in particular was lower than in the mannitol treatment.

Treating anthers with mannitol (0.3 M) along with cold stress (4°C) for 4 days in barley (Kruczkowska *et al.* 2002) or wheat (Hu and Kasha 1999) was ineffective. Kruczkowska *et al.* (2002) showed that with cultivars able to form numerous embryos/calli, mannitol pretreatment proves to be more efficient than the other tested pretreatments (chilling or jointly – chilling and mannitol). With recalcitrant genotypes, the frequency of obtained embryos/calli may be increased by chilling (4°C). However, it should not be assumed that a particular cold treatment would be appropriate for all genotypes as numerous apparent genotypic differences in anther response were reported in the literature (Shaeffer *et al.* 1979; Marsolais *et al.* 1984). The choice of the treatment method depends not only on the genotype, but also on the amount of material available for induction. With chilling, work may be distributed over a longer period than is possible with the mannitol technique.

Role of the gametocide

An original approach for changing the microspore status was applied by Picard *et al.* (1987) using a gametocide, applied to the donor plants. In this way they increased the embryoid induction 20-fold and obtained 10-fold more green regenerants. Vnutchkova and Tchebotareva (1990) obtained a two-fold increase in callus induction and green plant regeneration by employing the same method.

Role of culture conditions

Culture conditions play an important role in the *in vitro* response of wheat anthers. The most usually considered variables in incubation conditions are temperature and light. The effect of incubation temperature on the response of different genotypes was reported by Ouyang *et al.* (1983) and McGregor and McHughen (1990). In general, relatively high temperatures of 25-30°C were found to be optimal for both anther and pollen culture. Ouyang *et al.* (1983) investigated anther culture response to cultivation temperature of various cultivars, hybrids and lines. They found that the *in vitro* response of wheat anther culture to incubation temperature was very flexible. Decreasing the temperature by 2-3°C resulted in large variation in the frequency of pollen plants produced. Such an effect may often be observed at temperatures over 30°C or below 26°C. Xinias *et al.* (2001) observed a slightly better response of some genotypes at 32°C. Their results are in agreement with those reported by Marsolais *et al.* (1984) and Karimzadeh *et al.* (1995) who suggested that 30-35°C incubation temperature might be even more effective than 26-28°C. According to Xinias *et al.* (2001), a higher (32°C vs. 28°C) incubation temperature did not significantly modify the number of responding anthers or embryoids. They concluded that production of haploid plants is more dependent on the genotype than on the temperature regimes of anther cultivation. Ouyang *et al.* (1983) pointed out genotypic variation in the response to culture temperature between different cultivars. As regards the response to culture temperature, the most significant genotypic variation was found during continuous culture at 25°C and at 33°C for 8 days followed by culture at 25°C. For example, in cv. 'Kedong 58', an 8-day initial culture at 33°C resulted in a marked increase in the yields of pollen callus (11-fold) and green pollen plants (29-fold) over the control (continuous culture at 25°C). In another cultivar, 'Norin 10', however, the same treatment decreased the yields of pollen callus and green plants to 24% and 14% as compared to the control, respectively. Response to the 33°C treatment of anthers from the F_1 hybrid between these two cultivars was intermediate, suggesting that genotypic variation in response to culture temperature is a heritable characteristic.

In the study of Huang (1987) anthers were cultured at a wide range of different temperatures (between 15 and 40°C) for 8 days before all cultures were transferred to 25°C. The yield of calli increased with the treatment temperature, reaching a maximum at 30°C. Anthers cultured at 35°C for the first 8 days produced less calli, relative to those cultured at 30°C for the first 8 days, and those which were cultured at 25°C continuously. Culture of anthers at 40°C for 8 days was apparently deleterious.

Investigations on the influence of light on the induction stage of anther culture are scarce probably due to that light is thought to be dispensable for induction. Commonly, anthers are incubated in the dark or in dim, diffuse light. Bjornstad *et al*. (1989) showed suppressed embryoid formation when high light intensity (75 $\mu Em^{-2}s^{-1}$) was applied, while regeneration was stimulated in a recalcitrant genotype (cv. 'Runar'). Poor, diffuse light did not influence callus induction negatively and was found to even maintain a positive influence on regeneration. Ekiz and Konzak (1997) investigated the effects of light regimes on anther culture response in bread wheat. They established a negative influence on callus induction by high light intensity applied at the anther culture period. Jones and Petolino (1987) applied three light regimes but they could not observed significant differences between them. Ziegler *et al.* (1990) examined the influence of light (500 lux, 16 h) on anther culture ability and regeneration of green/albino plants of various spring wheat genotypes. Regeneration of green plantlets was improved by differentiation in the dark, whereas differentiation under light yielded more than 90% albino plants. When light was absent during the differentiation process, regeneration ability for green plants increased 7-fold. A possible explanation for the damage caused by light according to the authors might be a disturbance of carotenoid production and subsequent photooxidation of developing chlorophylls. The authors pointed out a positive influence of the absence of light in the first stages (10 days) of morphogenesis on the percentage of green regenerants. Microspores lacking protection from anther wall are more sensitive to light than when they are grown inside the anther. The sensitivity of microspores to light is more pronounced during the first ten days of culture.

Role of medium composition

A very important factor affecting androgenesis is medium composition. In particular, the concentration and combination of hormones play a certain role. In different experiments the following factors had been varied: macro- and microelements, vitamins, amino acids, activated charcoal, and different extracts (potato vs. carrot extract). The effect of medium on the rate of embryoid and plantlet formation from anther cultures has been studied by a number of researchers. Slightly modified Murashige and Skoog (MS) (1962) basal media with added growth regulators was used for the culture of excised anthers. For wheat anther culture two potato media were developed: Potato-1 (P-1) and Potato-2 (P-2) medium in the early 1970s (Research Group 301, Institute of Genetics, Academia Sinica 1976; Chuang *et al.* 1978). Both potato media, especially P-2, have been widely used in wheat anther culture. In each potato medium the basic part was the potato extract, and some defined inorganic and organic chemicals were added as supplements. In P-1 no inorganic salts (except iron) or vitamins were supplemented. In P-2 only half-strength of six major inorganic salts of a synthetic medium, W14 medium (developed in the Ouyang *et al.* (1989) lab), and one vitamin (thiamine), were added as supplements. In order to improve its utilization in the anther culture medium used, Ouyang *et al.* (2004) modified the synthetic medium W14 using 5% potato extract as an additive, but at the same time they decreased the amount of ingredients of W14 medium each to three-quarters of the original strengths. Their results showed that the efficiency of this potato-extract-containing medium was much better than that of the original synthetic medium. They also de-

monstrated that 5% potato extract was already a sufficient quantity for improving the efficiency of wheat anther medium. The authors suggested that the efficiency of any synthetic induction medium for wheat anther culture could be improved in this manner by adding 5% potato extract. El-Maksoud and Bedo (1993) studied P-2 and the modified N6 medium (MN6-medium) containing either sucrose (MN6+sucrose) or maltose (MN6+maltose) according to Chu *et al.* (1990) and they showed that P-2 induction medium was better. The effectiveness of P-2 was much better than that of N6 (Chu 1978) and other synthetic media and this was confirmed by a number of research teams (Shimada and Otani 1988; Ziegler *et al*. 1990; Cattaneo and Qiao 1991). In the experiments of Ziegler *et al.* (1990) medium P-2 was better than N6 with regard to the rate of responding anthers and embryoids produced in all four cultivars studied. The best results were obtained with cv. 'Nandu' where responding anthers were 0.8% on medium N6 and 3.7% on P-2. Karimzadeh *et al.* (1995) reported pronounced differences between the effects of various induction media. In their experiments, the chemically undefined P-2 (Chuang *et al.* 1978) medium gave best results for anther response and embryoid production, followed by the chemically defined W14 (Ouyang *et al.* 1989) medium. Otani and Shimada (1995) compared three kinds of synthetic media (C17 (Wang and Chen 1986), W14. and MN6) with P-2 for microspore-derived embryoid production in tetraploid wheat anther culture. All three synthetic liquid media contained 0.26 M maltose. Liquid P-2 medium contained 0.26 M sucrose. Liquid C17 medium was more effective than the other synthetic media and P-2, and thus this medium was considered as the proper medium for microspore-derived embryoid production in tetraploid wheat. Simonson *et al.* (1997) examined 5 different induction media and they observed significant differences in the embryoid initiation frequencies between them. Anther on P-1 initiation medium had the highest, whereas anthers on MS and on P-1 with MS salts (P-1/MS) media had the lowest embryoid initiation frequency (0.907 vs. 0.431 and 0.106, respectively). Slusarkiewicz-Jarzina and Ponitka (1997) investigated three different media: 1- P-2 medium and two MN6 media to which 90 g/l or 120 g/l sucrose was added (medium 2 and 3, respectively). They found that medium 1 was more efficient than media 2 and 3 for embryo induction in triticale, but green plants obtained on media 1, 2 and 3 were 12.5%, 9.9% and 1.3%, respectively. The number of green plants depended also on donor genotypes. The percentage of green plants from 20 genotypes was 2.7-28.7 on medium 1, 1.0-45.3 on medium 2 and 0.7-8.7 on medium 3. The rate of albino plants was high (69.5, 45.3 and 79.2 on media 1, 2 and 3, respectively).

Effect of carbohydrate type

The type of carbohydrate in the regeneration medium significantly influences the regeneration frequency of various wheat genotypes (Chu *et al.* 1990). Karsai and Bedo (1997) found that the type of carbohydrate applied in the regeneration medium had a significant effect, but it was strongly genotype-dependent. Sugar is known to function as a carbon source for cell growth as well as an osmotic regulator of the culture media (Zhou *et al.* 1991a; Ball *et al.* 1992; Otani and Shimada 1993; Karsai *et al.* 1994; Navarro-Alvarez *et al.* 1994; Scott *et al.* 1995). The normal level of sucrose is 2-4%; however, wheat anthers have been observed to grow better on media with 6-12% sucrose. This seems to be an osmotic effect rather than a need for a higher carbohydrate level. The effect of various sugars as a carbohydrate source of pollen embryo formation was extensively studied. P-2 with sucrose was estimated to be an effective medium for wheat anther culture. Slusarkiewicz-Jarzina and Ponitka (1997) found that sucrose concentration in the medium was an important factor inducing androgenesis and influencing the frequency of green plant regeneration in triticale. Media containing 9% sucrose were more suitable for androgenic response than those with 12% sucrose. In recent years many investigators preferred to use maltose in place of sucrose as a carbohydrate in the induction medium of wheat anther or microspore culture (Machii *et al.* 1998; Puolimatka and Pauk 1999; Liu *et al.* 2002). Sucrose is rapidly hydrolyzed to fructose and glucose which sharply increases the media osmolarity while no detectable osmotic changes occur in maltose-containing media. High osmolarity was regarded as a positive factor during the induction phase of culture (Zhou *et al.* 1991a; Ball *et al*. 1992) while the replacement of sucrose with maltose resulted in higher green plant regeneration in wheat (Orshinsky *et al.* 1990; Otani and Shimada 1993; Navarro-Alvarrez *et al*. 1994; Karsai *et al.* 1994; Otani and Shimada 1994). The frequency of pollen embryo production in wheat anther culture was reported to increase when sucrose was replaced by glucose (Chu *et al.* 1990) or maltose (Last and Brettel 1990; Orshinsky *et al.* 1990). Otani and Shimada (1993) investigated four monosaccharides (galactose, glucose, manose and fructose) and 4 disaccharides (sucrose, maltose, lactose and cellobiose). Liquid P-2 (Chuang *et al.* 1978) with sucrose (90 g/l) as a carbohydrate source was also used to compare with C17 medium (Wang and Chen 1986) with maltose (0.26 M). In all cultivars studied, the largest number of pollen embryos was formed on medium containing maltose among the media containing various sugars. Pollen embryos produced in maltose-containing medium showed more vigorous green plant regeneration than those in media containing other kinds of sugars. The authors proposed 0.26 M maltose as the appropriate concentration for wheat pollen embryo production. They established that the appropriate medium for pollen embryo formation in wheat anther culture differed with cultivar. The same results were observed in the anther culture of tetraploid wheat (Otani and Shimada 1994, 1995). In their experiment, the frequency of green plant regeneration was highest for pollen embryos produced on C17 medium containing maltose (0.26 M). However, albino plant formation frequency was also highest in pollen embryos from maltose-containing medium. These suggest that maltose has an effect on plant regeneration but not on albinism in wheat anther culture. El-Maksoud *et al.* (1993) also found that the MN6 induction medium + sucrose gave a better anther response and embryoid induction frequencies than MN6 induction medium + maltose. However, they did not observe a significant difference for green and albino plantlet induction when the MN6 regeneration medium contained either sucrose or maltose. Chu *et al.* (1990) found that the carbohydrate composition of the regeneration medium significantly influenced the regeneration frequencies of various wheat genotypes. In the study of Karsai and Bedo (1997) maltose concentration in the induction medium significantly influenced the anther culture efficiency in triticale but the genotype and the genotype × maltose interaction were also significant factors. In most cases the application of 0.21 or 0.26 M maltose resulted in higher embryo induction while a further increase in the maltose concentration to 0.31 M had no further enhancing effect. According to Karsai *et al.* (1994) a higher maltose concentration significantly improved the embryoid induction percentage of three triticale genotypes studied but it did not lead to a consistent increase in green plant regeneration. Maltose at 0.26 M vs. 0.21 M led to higher green plant regeneration only in the case of wheat. Green plant regeneration is the final expression of a large number of interacting factors, including embryoid induction and the frequency of albinism (Karsai *et al.* 1994). While embryo induction is highly heritable, plant regeneration seems to be a more complex trait because of its great sensitivity to environmental factors (Balatero *et al.* 1995). Kovacsne and Pepo (2006) examined the effect of four sucrose concentrations (45, 60, 75 and 90 g/l) and four maltose concentrations (65, 100, 135 and 170 g/l) on callus induction, plant regeneration and green plant proportions. The authors established that the callus induction response for cv. 'Pavon 76' to sucrose was higher than to maltose, regardless of their concentrations. The callus induction increased dra-

matically for both induction media when either sucrose or maltose were increased from 45 to 90 g/l and from 65 to 170 g/l, respectively. The plant regeneration was 50.4-51.4% in medium containing various amounts of sucrose and 49.8-48.0% for that with maltose. The proportion of green plants was highest for sucrose at 90 g/l (22.3%) and for maltose – at 170 g/l (31.7%). In the study of Trottier *et al.* (1993) the effect of replacing sucrose by maltose in wheat anther culture varied with the genotypes of the anther donor plants. Marciniak *et al.* (1998) investigated the effect of genotype, medium and sugar on triticale anther culture response. They tested two sugar versions on P-2 and C17 media, containing 9% of either maltose or sucrose. Their results showed significant differences between genotypes for all the investigated traits. Seven out of ten genotypes developed green plants from embryoids induced only on maltose-containing media, whereas one genotype gave green plants only on sucrose containing media. P-2 with maltose yielded the highest number of green plants in more genotypes than either of the remaining media. The genotype × medium, genotype × sugar and medium × sugar interactions were significant, evidence that modification of the induction media can be one way to further increase the anther culture efficiency of cereals. Gonzalez and Jouve (2000) used 4 induction media to induce embryogenesis in 3 triticale genotypes and their F_1 hybrids. They established that the induction media which contained 2 mg/l 2,4-D and 50 g/l sucrose plus 50 g/l maltose or 100 g/l maltose, respectively, were the best at inducing embryogenesis, although strong genotype differences were found.

The response of isolated microspores to sugar is different from that of cultured anthers. For example, anthers incubated on medium containing sucrose or fructose developed embryos and the optimal concentration for embryogenesis from anthers was 10, 20 and 5 mM of sucrose, glucose and fructose, respectively (Finnie *et al.* 1989). However, Scott *et al.* (1994) showed that microspores incubated in the presence of different concentrations of sucrose, glucose and fructose (from 5 to 175 mM) died during the early stages of incubation. Moreover, microspores also died, when incubated in the presence of a combination of 175 mM maltose with varying concentrations of sucrose, glucose or fructose. In contrast, microspores developed into embryoids or calluses when incubated in the presence of maltose as the sole carbohydrate source and the optimal concentration of maltose was 175 mM. The authors concluded that sucrose, glucose, and fructose have a toxic effect upon cultured cells, which is exerted in the presence or absence of maltose. It remains unclear, however, whether the toxic effect is due to the sugars themselves, or to some metabolic product of the cells. The differences between the response of isolated microspores and anthers to sugar could be accounted for by the anther wall acting as a barrier between the microspores and the culture medium.

The role of osmotic potential

As was already mentioned, sugar has two functions in culture media: it serves as a carbon source and as an osmotic regulator. Since the sugar content of media was found to change little during the culture period, it may be the major osmotic regulator in the medium. The osmotic potential of the induction medium plays a significant role in the anther culture response. It was also found to have a significant effect on the green plant regeneration of wheat (Finnie *et al.* 1989; Zhou *et al.* 1991a). According to Kovacsne and Pepo (2006) the difference in the percentage of green plant yield between maltose concentrations demonstrates the effect of the osmotic potential of the medium on albinism. The role of osmotic potential in the anther culture response in cereals was first demonstrated when the sucrose content of the medium was increased to 0.26 M from the widely-used level of 0.06-0.09 M in somatic tissue culture (Ouyang 1986). A further increase was achieved when sucrose was replaced by maltose (Zhou *et al.* 1991a). These changes in the induction medium resulted in higher embryo induction together with increased green plant regeneration both in wheat (Zhou *et al.* 1991a) and barley (Kuhlmann and Foroughi-Wehr 1989). A reason for this was the difference in the hydrolysation dynamics of these two carbohydrates; sucrose is rapidly hydrolysed to fructose and glucose, increasing the medium osmolarity during culture, whereas no detectable osmotic changes occur in medium containing maltose (Kuhlmann and Foroughi-Wehr 1989; Zhou *et al.* 1991a). According to Kovacsne and Pepo (2006) many of the reported effects of medium modifications and pretreatments may be related to osmotic potential and if the hypothesis about the importance of the osmotic potential of the medium is correct, more attention should be paid to establishing the optimal osmotic potential for regeneration media. On the other hand, the optimal osmotic potential for certain genotypes may not have been identified because this value may be highly specific for individual genotypes (Zhou *et al*. 1991a)

The nutritional requirements of excised anthers are much simpler than those of isolated microspores. In isolated microspores it is obvious that certain factors responsible for the induction of androgenesis, which may have been provided by the anther, are missing, and that these have to be provided through the medium.

Effects of plant growth regulators

The type and concentration of growth regulators (hormones) in the induction nutrient medium have a basic role for the change in the path of microspore development from gametophytic to sporophytic. Use of various PGRs has been shown to modify callus growth and plant regeneration rates. The induction of microspore cell division and cell proliferation *in vitro* is achieved by auxins (2,4-D, α-naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA)), and the most potent one in this process the synthetic growth regulator: 2,4-D. In cereals, the synthetic auxin 2,4-D is a preferred auxin for the induction of somatic embryogenesis and androgenesis *in vitro.* Depending on its concentration in the medium, 2,4-D may act as a stress factor contributing to a change in the developmental path of pollen or as a component needed for embryo development. Hoekstra *et al*. (1996) showed that a high concentration of 2,4-D, e.g. 20 mg/l, can result in obtaining green plants in barley anther culture without pretreatment. In *in vitro* culture of anthers pretreated with low temperature or with mannitol, the preferred 2,4-D concentration in the medium varies greatly with different authors: from 0.2 mg/l (Hoekstra *et al*. 1996) up to 8 mg/l (Marsolais and Kasha 1985). A higher 2,4-D concentration inhibits direct embryogenesis and promotes callus growth.

Kruczkowska *et al.* (2005) investigated the effect of various 2,4-D concentrations on the androgenic response and plant development in anther culture of barley. They showed that osmotic stress can not be replaced with a high (20 mg/l) 2,4-D concentration. Most embryos, embryogenic calli and plants (including green plants) were obtained with 0.7 mg/l mannitol pretreatment and 0.2 mg/l of 2,4-D in the induction medium. Auxin concentration also affected the dynamics of embryogenic structure formation and plant development from embryogenic structures. Concentrations higher than 0.2 mg/l slowed down embryogenic structure formation and plant development and increased the proportion of calli compared to embryos in plant regeneration. As a result, the frequency of androgenesis was reduced. According to Gorbunova *et al.* (1993) and Kruglova and Gorbunova (1997) the type and concentration of PGRs in the induction medium changed the ratio between calluses and embryoids considerably. When 2,4-D was included in the nutrient medium mainly callus was induced, while in the presence of NAA or IAA the rate of embryoids increased (Konzak and Zhou 1991). From the geneticist's point of view the direct formation of embryos from pollen is preferable to the indirect formation of embryos via callus since the callus derived plants are mostly undesirable as they ex-

hibit genetic variations and polysomy (Nitsch 1977; Bajaj 1990).

The response to 2,4-D concentration depends not only on the cultivar, but also on the method (with or without pretreatment) (Kruczkowska *et al.* 2005). The authors showed that pretreatment is more effective and the response to 2,4-D concentration depended on the concentration of mannitol used during the pretreatment.

Induction may also be affected by other stress-causing components of the medium (Indrianto *et al.* 1999). Some authors substituted other auxins for 2,4-D in the androgenesis-inducing medium (Sozinov *et al.* 1981; Brown *et al.* 1989; Shimada and Otani 1989; Hassawi *et al.* 1990; Szarejko 1991, 2003). Sozinov *et al.* (1981) established that the addition of proline (100-160 mg/l) and oxyproline (100-160 mg/l) to the initial medium promoted the appearance of embryoids from calluses and further plant regeneration from them. Abscisic acid (ABA), is a plant hormone which has several effects on the development of embryos. ABA influences cellular differentiation that affects both the anatomy and biochemistry of the explant (Ammirato 1988). Promotion of embryogenic callus and increased plantlet regeneration were reported in the presence of ABA and ABA analogs in wheat (Qureshi *et al.* 1989). When applied at low concentrations, ABA was found to increase embryogenesis in wheat (Brown *et al.* 1989). Shimada and Otani (1989) observed a stimulating effect of low concentrations of ABA (0.1-0.5 mg/l) on embryo formation in some poorly responding genotypes while in others the result was negative. Hassawi *et al.* (1990) examined seven auxins (2 mg/l) in combination with 1 mg/l kinetin as well as four cytokinins (1.5 mg/l) with NAA (0.75 mg/l). The best results for callus induction were obtained on media with 2,4-D, picloram and dicamba. In an experiment by Szarejko (1991) an attempt to substitute other auxins for 2,4-D was unsuccessful in the anther culture of barley, but in later publications (Cai *et al.* 1992; Szarejko 2003) she recommended the combination of NAA and benzylaminopurine (BAP) in the induction medium. Chu and Hill (1988) also found advantageous the addition of NAA (1 mg/l) in combination with amino acids in the induction medium.

Moieni and Sarrafi (1996a) investigated the effect of gibberellic acid (GA_3), β-phenylethylamine (PEA), 2,4-D and genotype on androgenesis in hexaploid wheat (*T. aestivum*). In two genotypes out of three studied, an antagonistic effect between GA_3 and 2,4-D on embryo yield was observed. Replacing 2,4-D (0.5 mg/l) by either GA_3 (0.385 mg/l) or PEA (7.750 mg/l) in the medium decreased the number of embryoids and regenerated plantlets per 100 anthers. PEA increased the number of embryoids per 100 anthers in two genotypes ('BP40' and 'BP25' pure lines of *T. aestivum*). Basal medium supplemented with GA_3 increased the ratio of plant regeneration in comparison with the control basal medium in one out of three genotypes tested. Haploid plants were produced at a high frequency in induction medium supplemented with GA_3 (with or without 2,4-D) (69.26 and 14.89%, respectively). The results showed that GA_3 alone or together with 2,4-D had a very important effect on direct plant regeneration.

Kruczkowska *et al.* (2005) tested three auxins (2,4-D, picloram and dicamba) used in various proportions and only combinations with 2,4-D brought favorable results. Kremenevskaya *et al*. (2004) treated anther donor plants with kinetin (4 mg/l) at early development stages in a secalotricum line 'Paparatz × AD60' and they showed that callus and embryoid yield increased by approximately 1.7 times more than the control (6.8% vs. 3.96%). There is an assumption (Reinert and Bajaj 1977) that the requirement for auxin and cytokinin depends on their endogenous levels in anthers. Media rich in PGRs encourage the proliferation of tissues other than microspores (i.e. anther wall, connective and filament) and should be avoided, because in such cases mixed calli with cells of different ploidy levels are obtained.

Ghaemi *et al.* (1994) examined whether the addition of silver nitrate, colchicine and cupric sulfate to the medium would increase the number of microspore-derived embryoids in tetraploid wheat. Three different concentrations of silver nitrate (1, 2.5 and 5 mg/l), cupric sulfate (2.5 and 10 mg/l), or colchicine (10, 100 and 200 mg/l) were used in their experiments. The presence of silver nitrate (2.5 and 5 mg/l) and cupric sulfate (10 mg/l) usually increased the frequency of embryoid formation in 3 genotypes out of the 4 studied. On the contrary, colchicine had a significant negative effect on anther culture responses for 3 out of the four genotypes studied. Lachermes (1992) also found that the addition of silver nitrate in anther culture media improved embryo production in wheat. Ghaemi *et al.* (1994) explained the positive effect of silver nitrate on pollen embryogenesis with the blocking of the inhibitory effect of endogenous ethylene on the embryo. That silver is an ethylene inhibitor and stimulate morphogenesis in tissue cultures was shown by Purnhauser *et al.* (1987). The presence of silver nitrate and cupric sulfate only slightly increased the frequency of microspore-derived embryoid formation.

The concentration of organic and inorganic salts and PGRs are changed in the media in order to modify morphology and callus production, and this depends on the genotype (Zhou and Konzak 1989; Hassawi *et al.* 1990; Rafi *et al.* 1995; Gonzalez and Jouve 2000). The balance of ions in the nutrient medium significantly influences the quality of the induced structures. Iron in the medium plays a very important role and is indispensable (Reinert and Bajaj 1977). Feng and Ouyang (1988) pointed out that the frequency of callus induction and green plant regeneration increased when the concentration of KNO_3 was increased to 15 mM (1500 mg/l) in the induction medium. According to the authors, the influence of KNO_3 on green plant regeneration is due to the K^+ and NO_3^- ions, while in the medium with too high a KNO_3 concentration callus induction decreased due to the NO_3^-. In N6 nutrient medium (Chu 1978), the NO_3^- /NHH_4^+ ratio is highly increased because a low concentration of NH_4^+ ions favors embryo formation. A low level of ammonium nitrate and high level of glutamine in the medium resulted in direct pollen embryogenesis in barley (Datta and Wenzel 1988).

Henry and de Buyser (1981) replaced potato extract with an amino acid and in this way they increased the percentage of embryo formation. In their experiment, in the best medium (containing glutamine, 0.5 g/l) more than 75% of the spikes were embryogenic. A higher concentration of glutamine was toxic for androgenesis. Zhu *et al.* (1990) examined the effects of amino acids on callus differentiation in barley anther culture. They established that supplements of 8 mg/l L-Alanine, L-Asparagine, L-Glutamine and L-Alanine+L-Asparagine to modified MS media increased the frequency of differentiation and the percentage of green plant regeneration in barley callus. It also decreased the ratio of albino to green plants. The total frequency of differentiation for the various media was from 55.6 to 74.59% and percentage of green plants – from 11.80 to 14.52%, compared with the control media (48.74 and 6.73%, respectively). The authors observed a strong correlation of isozyme patterns and peroxidase activity with differentiation and green plant regeneration. They suggest that amino acids stimulated or inhibited the related peroxidase genes, or amino acids firstly stimulated cell differentiation which then influenced peroxidase genes. Chu and Hill (1988) optimized embryo development by a set of amino acids (serine, proline, arginine, aspartic acid and alanine at 40 mg/l each, and glutamine at 400 mg/l). They observed that amino acids supplementation benefited not only pollen embryoid frequency, but also plant regeneration. Trottier *et al.* (1993) established a stimulating effect of 19 combinations of amino acids, but when they added maltose, the positive influence was eliminated. The inclusion of glutamine did not increase embryo induction and plant regeneration in the experiments of Puolimatka and Pauk (2000).

However, despite the positive influence of amino acids, their use in nutrient media is too expensive for routine technology.

Effect of the physical state of the nutrient medium

The physical state of the nutrient medium also influenced the induction of embryogenic structures and regeneration. Chu and Hill (1988) did not observe significant differences in the induction of embryoids on liquid and solid nutrient media. Cistue *et al.* (1994) showed that there were no significant differences between liquid and solid media with mannitol. Jones and Petolino (1988) reached a two-fold increase in embryoid structures on liquid medium but they had significantly lower regeneration potential. Zhou and Konzak (1989) also obtained high callus induction on liquid medium but low regeneration frequency. In their experiment callus induction was 10 to 20 times greater for anthers cultured on liquid medium compared to agar-solidified (6 g/l) induction media. In the study of Ghaemi *et al*. (1995a) liquid medium was more efficient than solid medium for embryogenesis of tetraploid wheat. Embryoids from liquid medium produced fewer albino plantlets than from solid medium in their experiment, which was in contrast with the results of Jones and Petolino (1988) for hexaploid wheat. The increase of embryoid production by liquid induction medium in their experiment is in agreement with a previous report for hexaploid wheat (Zhou *et al*. 1991b). The results of Ghaemi *et al*. (1995a) are in agreement with those of Lazar *et al*. (1985) who reported that cold pretreatment at 4°C followed by cultivation in liquid medium increased the regeneration ability. Later, Lazar *et al.* (1990) investigated the influence of agar concentration on embryoid induction in six wheat cultivars and they did not observe significant differences between liquid and semi-solid (0.4% agar) medium. With an increasing in agar concentration to 1.5%, embryoid formation decreased significantly in all genotypes studied. Similar results were obtained by McGregor and McHughen (1990) on liquid and solidified (0.8, 1.8% agar) nutrient medium. In the experiment of Ziegler *et al.* (1990), induction medium P-2 in solid, semisolid, and liquid form (3, 2, 1 and 0 g/l agarose, respectively) was shown to increase the anther response, embryoid induction and plant regeneration with the decrease in the agarose content. Karsai *et al.* (1994) found that induction medium containing agar was more effective than liquid medium, while Puolimatka and Pauk (2000) did not observe difference between liquid and solid medium concerning the androgenic reaction. Stober and Hess (1997) could increase the number of embryoids and regenerants on liquid medium. The change in the concentration of the gelling agent had positively influenced embryoid induction but had no effect on the regeneration of green or albino plants. In the study of Ghaemi *et al.* (1995a) liquid medium improved total regeneration when compared with solid medium (4 g/l gelrite) from 4.04 to 7.68%.

Further improvements in the efficiency of anther culture were obtained by increasing the liquid medium viscosity through the addition of Ficoll, a neutral, highly branched, high-mass, hydrophilic polysaccharide (Kao 1981; Kuhlman and Foroughi-Wehr 1989; Zhou and Konzak 1989). In the study of Ghaemi *et al.* (1993) the use of Ficoll and maltose did not improve green plantlet production. Charmet and Bernard (1984) reported higher triticale embryoid induction in Ficoll-containing media. The differences between the various gelling agents were modest. This was particularly true for plant regeneration. In terms of embryoid induction, agar medium was inferior to liquid or Ficoll-containing media. In terms of green plant regeneration, liquid induction medium was inferior to media containing Ficoll. The positive influence of Ficoll on the androgenic reaction was pointed out by Zhou and Konzak (1989, 1997), Kuhlman and Foroughi-Wehr (1989), Fadel and Wenzel (1990), Yuan *et al.* (1990), Zhou *et al*. (1992) and Karsai *et al.* (1994). Zhou and Konzak (1989) improved the androgenetic haploid induction in spring wheat by adding 200 g/l Ficoll. In the study of Kuhlman and Foroughi-Wehr (1989) optimal embryo and callus formation was obtained on liquid medium with 20% Ficoll and 20 g/l maltose. In the study of Fadel and Wenzel (1990), liquid P-2 medium with 100 g/l Ficoll was the most suitable medium for androgenetic pollen development, although significant genotypic differences were observed. Zhou *et al*. (1992) also found that 100 g/l Ficoll was better than 200 g/l. Higher concentrations were found to be deleterious. Yuan *et al.* (1990) obtained 1.5 times more calli on 10% Ficoll-containing medium, compared to medium without Ficoll. In wheat and triticale, induction media with agar (0.6%) and Ficoll (10%) were superior to the liquid form (Karsai *et al.* 1994). Among the three induction media studied by Zhou and Konzak (1997) the liquid and Ficoll (100 g/l)-containing media produced significantly more calli than the agar-solidified (6 g/l) medium for all five wheat genotypes. The addition of Ficoll increased plant regeneration in four out of five genotypes and the green plant percentages in two genotypes. The liquid and Ficoll-containing media produced more green plants per 100 anthers than the agar-solidified medium. Significant interactions between genotypes and induction media were observed for callus induction, plant regeneration and green plant yield. It was concluded that the physical conditions of the induction media exerted differential influences on anther culture responses for different genotypes.

The low level of pollen embryo formation in solid medium could be due to the inhibitory effect of Gelrite (Ghaemi *et al.* 1995a). Another reason for the low embryoid yield in solid medium could be the fact that pollen-derived embryoids remain within the anther wall in solid medium, while they float freely and have more direct access to nutrients in liquid medium. Although liquid medium may allow for enhanced nutrient distribution, it also exposes a substantial proportion of the embryoids to anaerobic conditions, leading to reduced plant regeneration (Kuhlman and Foroughi-Wehr 1989; Zhou and Konzak 1989).

Zhou and Konzak (1997) examined the effects of five starch forms (3%) and three gelling agents (0.2%) on the induction of androgenesis. In terms of green plant yield liquid medium produced the highest green plant percentage, but commercial wheat starch and potato starches, and gelrite-containing media were as good as the liquid medium, indicating that starch could potentially replace agar and Ficoll as an induction medium component. According to the authors, some starch at low concentrations may offer unique properties as the medium component/gelling agent for use in anther culture.

The induction of embryogenic structures can be influenced by the addition of charcoal in the induction medium (Jones and Petolino 1988) or by other specific factors (i.e. cold sterilization of nutrient medium (Chu and Hill 1988; Otani and Shimada 1993), low pH (Ghaemi *et al.* 1994), density of anthers (Chu and Hill 1988), etc. Incorporation of activated charcoal (AC) into the medium stimulated the induction of androgenesis in tobacco anthers (Bajaj 1976). It was shown that the percentage of androgenic anthers could be raised from 41 to 91 by the addition of AC (2%) to the medium. However, it seems that AC can also cause small increases in the induction of diploid plants. At present, no satisfactory explanation can be given for the stimulatory effect of AC. Perhaps, it might be responsible for the removal of inhibitory substances from the medium. However, it seems more likely that the level of PGRs, both endogenous and exogenous, is regulated by absorption onto AC (Bajaj 1976; Chu and Hill 1988).

Effect of medium pH

The effect of medium pH proved to be crucial in several *in vitro* processes (Basu *et al.* 1988; Cousson *et al.* 1989; Smith and Krikorian 1990), but very little information is available concerning the effect of changes in the pH of the induction medium on anther culture of cereals. In experiments on *in vitro* aluminum Al^{3+} selection in wheat anther culture, Kovács *et al.* (1993) found that decreasing the pH

from the widely used value of 5.8 to 4-4.5 had an enhancing effect on anther culture efficiency. Karsai *et al.* (1994) studied the effect of the pH of the induction medium and maltose concentration on *in vitro* androgenesis of hexaploid winter triticale and wheat. They established that changes in pH had no effect on triticale varieties, while this treatment significantly increased embryoid induction in a wheat variety at pH 4.8. Their results supported the observations of Kovács *et al.* (1993) who showed that a reduction in pH led to a significant increase in embryoid induction and green plant regeneration in wheat. In contrast, low pH had a negligible effect on the *in vitro* androgenesis of triticale genotypes. An enhancing effect of low pH was also reported by Smith and Krikorian (1990). They found that in carrot callus culture the maintenance and propagation of embryogenic tissues at the proembryo stage was increased at pH 4.0. The influence by low pH on the efficiency of anther culture and the significant difference between the responses of wheat and triticale genotypes are interesting themes for future investigations.

FACTORS AFFECTING PLANT REGENERATION

Regeneration of plants from callus is achieved by a change in the hormone component or by the omission of hormones. The level of sucrose is also invariably reduced to 2-3% in regeneration media. Initially, the regeneration nutrient media contained a full set of salts and vitamins of the MS medium, the quantity of 2,4-D was reduced or absent, the concentration of carbon source decreased to 3% and in the presence of kinetin, NAA (Chu *et al.* 1973; Wang *et al.* 1973) or IAA (Ouyang *et al.* 1973) were added. Generally, the regeneration process is less sensitive to the nutrient medium composition. That is why Zhuang and Jia (1983) developed a simpler and more effective regeneration medium 190-2, containing 0.9 M sucrose. In the study of El-Maksoud and Bedo (1993) the regeneration media used were 190-2 and MN6 containing either sucrose or maltose. They found that better plantlet regeneration occurred on 190-2. The green plantlets per 100 anthers varied from 4.49 to 9.55 for cultivars on 190-2 regeneration medium, from 0.00 to 3.25 on MN6+sucrose and from 0.00 to 4.21 on MN6+ maltose. In the study of Karsai and Bedo (1997) of three basal regeneration media (MS, 190-2 and MN6), those based on 190-2 and MN6 were shown to be more efficient with strong genotype dependence. Zhou and Konzak (1989) improved the regeneration potential of calluses derived from liquid medium replacing the PGRs in the original 190-2 by IBA and GA_3. In some cases regeneration improved with the complete absence of PGRs (Konzak and Zhou 1991). Gonzalez and Jouve (2000) also found that medium without hormones was the most successful with respect to plantlet regeneration.

As was already mentioned, plant regeneration is a complex trait since it is sensitive to environmental factors. The carbohydrate composition of the regeneration medium significantly influenced the regeneration frequencies of various wheat genotypes (Chu *et al.* 1990; Karsai and Bedo 1997). The changes in carbohydrate composition of the regeneration media affected similarly all 14 wheat varieties studied by Chu *et al.* (1990), while in triticale the effect of carbohydrate type applied to regeneration medium was strongly genotype-dependent (Karsai and Bedo 1997). Karimzadeh *et al.* (1995) also indicated that both androgenic response and regeneration ability were greatly genotype dependent and their regulation was genetically independent. Using wheat/rye chromosome addition lines, Lazar *et al.* (1987) found that only few individual rye chromosomes, especially chromosomes 4R and 1R, had an enhancing effect on green plant regeneration.

Another factor influencing plant regeneration is light. Ziegler *et al.* (1990) pointed out a positive influence of the absence of light in the first stages of morphogenesis on the percentage of green regenerants. In the absence of light, regeneration ability for green plants increased 7-fold. In the study of Bjornstad *et al.* (1989) high light intensity (75 $\mu Em^{-2}s^{-1}$) during the induction phase strongly suppressed callus induction, but stimulated green plant regeneration.

Regeneration can occur in a wider temperature interval compared to embryo induction, but commonly the calluses and embryoids are cultivated at 25°C in the light and appropriate photoperiod. To stimulate rooting of regenerants liquid medium containing IAA (1 mg/l), kinetin (1 mg/l) and glutamine (146 mg/l) (Lu *et al.* 1991) were used.

Puolimatka and Pauk (2000) observed that the duration of incubation had a significant effect on the regeneration capacity of embryogenic structures and that the highest percentage of regenerants was produced 6-7 weeks after cultivation of anthers. When the embryogenic structures remained too long on the induction medium before being transferred to the regeneration medium, regeneration decreased (Ouyang *et al.* 1983; Chu and Hill 1988; Ball *et al.* 1993).

ALBINISM

A big problem related to effectiveness of anther culture in cereals is that a great number of albino regenerants are obtained. These are due to genetic reasons as well as to the method of anther cultivation. Tuvesson *et al.* (1989) explained the regeneration of albino plants by the action of nuclear genes. Wheat-rye translocation 1BL-1RS increased production of green regenerants (Henry and de Buyser 1985; Cattaneo *et al.* 1991; Tuvesson *et al.* 1991) due to gametophytic genes located on the rye chromosome arm 1RS (Henry *et al.* 1993). According to Day and Ellis (1984, 1985) perhaps a deletion in the chloroplast DNA, which is apparent in *in vitro* cultivation, is a reason for albinism. Mouritzen and Holm (1994) established plastid genomic deletions in the early stages of regeneration. Caredda *et al.* (2000) concluded that the regeneration of albino plants is the result of degradation of plastid DNA, explaining why plastids are unable to develop into chloroplasts. According to Torp *et al*. (2001) genes on the chromosomes affecting albino plant formation may exert their effect via interaction with events in plastid development. Mosgova *et al.* (2005, 2006) studied the possible mechanisms of albinism in wheat anther culture. They examined two evolutionary unstable regions of the chloroplast genome in dihaploid wheat. These regions include some genes, changes in which could be associated with albinism in anther culture. Using PCR, they showed that *atpB* was the gene most often not detected in the lines examined. The authors suggested that regeneration of albino plants is accompanied by the deletion of a chloroplast DNA region harboring this gene. Another reason for the appearance of albino regenerants, according to Aubry *et al.* (1989), could be the changes of the molecule organization of the mitochondrial genome.

According to Ouyang *et al.* (2004) the albino shoot differentiation frequency is much more relative to anther donor genotype than to medium composition. In their experiment three different media were tested: W14 (Ouyang *et al.* 1988), PW14 (W14 with 5% potato extract), and 3/4W14 (with the salts (except iron) and vitamins (including glycine) reduced each to ¾ strength of the original W14 medium). The authors showed that there were no significant differences in the frequency of albino shoot differentiation between the three different media, although the frequency of green shoot differentiation differed significantly. These results indicated that medium components affecting the frequency of green shoot differentiation will not affect albino shoot differentiation as well. This also implies that the frequency of albino shoot differentiation is not as sensitive to the variation of induction medium components as green shoot differentiation. Comparing three different culture media (P-2, W14 and N601 (Liang *et al.* 1987)), Karimzadeh *et al.* (1995) established that only W14 medium gave a satisfactory high green plant regeneration percentage and green/albino ratio. The highest green plant frequency and green/albino ratio (82.6/20.0) was for this culture

medium in the case of 'Mw15' genotype.

However, having in mind the genotypic specificity, albinism can be strengthened or weekend depending on a number of other factors. In the study of Andersen *et al.* (1988) donor plants growing in the field had a better androgenic reaction than those, growing in the greenhouse, but they obtained a great percentage (8.8%) of albino regenerants. Anther culture conditions were found to have a strong effect on the degree of albinism in cereal pollen plants. The effect of temperature on regeneration potential for green plants was examined by Huang (1987). The results indicated an increased ratio of green/albino pollen plants with the increase of the initial culture temperature. The most dramatic difference in regeneration potential for green plants was between the 30 and 35°C treatment. Three times as many calli from anthers cultured at 35°C regenerated green plants relative to those, cultured at 30 or 25°C. The effect of temperature on albinism, as well as the mechanism of albinism is complicated. In wheat anthers cultured at higher temperatures, pollen calli appeared and were transferred earlier to the regeneration medium (Huang 1990). According to the author, it is highly possible that some components (e.g. high level of sucrose and/or auxin) of the culture medium used for callus production, have an inhibitory effect to plastid development and the longer the pollen is maintained in this medium, the more likely it is that the plastids will malfunction.

Ziegler *et al.* (1990) pointed out that regeneration of green plantlets was improved by differentiation in the dark, whereas differentiation under light (500 lux, 16 h) yielded more than 90% albino plants. The authors explain the damage caused by light with a possible disturbance of carotenoid production and subsequent photooxidation of developing chlorophylls.

The type and concentration of carbohydrate source and medium osmolarity also affect albinism. Karsai and Bedo (1997) showed that in spite of the strong genotype effect on the anther culture of triticales, the application of 0.26 M maltose proved to be the best for the green plant production of all the varieties studied. In the study of Kovacsne and Pepo (2006) the differences in green plant percentages between maltose concentrations demonstrated the effect of medium osmolarity on albinism. The green plant proportion showed a linear response with concentration and was highest at 90 g/l for sucrose (22.3%) and at 170 g/l for maltose (31%).

Amino acid supplements also exert influence upon green plant percentage and the green/albino plant ratio. Supplements of 8 mg/l L-Alanine, L-Asparagine, L-Glutamine and L-Alanine+L-Asparagine to MS media increased the green/albino ratio (Zhu *et al.* (1990). Except for medium with L-Alanine (2.87), the ratio of albino to green plants in media with added amino acids decreased in order down to 2.13, 1.42 and 1.39, respectively, from 2.75 in the control.

GENOTYPE-ENVIRONMENT INTERACTION

Many authors (Lazar *et al.* 1984a; Jones and Petolino 1987; Fadel and Wenzel 1990; Lazar *et al.* 1990) pointed out that the influence of genotype was paralleled by the genotype-environment interaction. The existence of numerous uncontrolled factors induced by environmental variations (Ockendon and Suntherland 1987; Kasha *et al.* 1990) makes it difficult to study the *in vitro* response inheritance in anther cultures. In the study of El-Maksoud and Bedo (1993) the genotype × medium interaction was only significant for responding anthers and for induction of embryoids but was not significant in the case of green plantlet induction. The effect of genotype, liquid and solid medium, ancymidol in liquid medium, spike cold pretreatment and their interaction was investigated by Ghaemi *et al.* (1995a) in tetraploid wheat. They found that the effect of genotype and medium was significant for the traits studied, but their interaction was only significant for embryoid production and total regeneration. The interaction between all studied variables "genotype × medium × cold pretreatment" (G×M×CP) was significant for all traits studied except for green plant regeneration per 100 embryoids. The results demonstrated a strong interaction between (G×M×CP) and showed that anther culture in liquid medium without cold pretreatment was more suitable for a genotype which could express its full potential in these conditions. Karsai and Bedo (1997) found that in triticale the genotype × maltose interactions were highly significant factors. They examined 5 triticale genotypes and their interaction with different maltose concentrations. The embryoid induction of 3 out of the 5 varieties studied increased significantly when 0.26 M maltose was applied instead of 0.21 M. Compared to embryoid induction, there was an even greater difference between the varieties in the responses of total plant regeneration and green plant regeneration to these maltose concentrations. Karimzadeh *et al.* (1995) studied the effect of the interaction between low temperature, different nutrient media and genotype on the androgenic capacity of winter wheat cultivars. They found significant differences between the two genotypes studied ('Mw06-83' and 'Mw15') and also among the effects of the various induction media. The chemically undefined P-2 gave the best results for embryoid induction, followed by the chemically defined W14. Cold treatment (at 4°C) had no modifying effect on total plant regeneration. Interactions genotype × sugar and medium × sugar were observed by Marciniak *et al.* (1998). They examined the androgenic response of 16 triticale genotypes on two induction media (P-2 and C17), containing 9% of either maltose or sucrose. There were significant differences between genotypes for all the investigated traits. Maltose was superior to sucrose (on average respectively 0.69 and 0.03 green plants per 100 anthers). P-2 with maltose yielded the highest number of green plants in more genotypes than either of the remaining media. Zhou and Konzak (1997) observed significant interactions between genotype and induction media for callus induction, plant regeneration and green plant yield in wheat. Genotype × nutrient medium interaction was established by many other authors as well (Liang *et al.* 1987; Lasar *et al.* 1990; Chaplicki 1993; Otani and Shimada 1994, 1995; Moieni and Sarrafi 1996b). It was concluded that the modification of nutrient medium may increase both embryoid number and green plant regeneration in different cultivars. Jones and Petolino studied the effect of donor plant growth temperature and photoperiod on embryo formation and plant regeneration from cultured anthers in 5 genotypes of wheat (*T. aestivum*). Significant genotype and genotype × environment interactions for embryo formation were established. Studying a series of reciprocally substituted alloplasmic lines, Orlov *et al*. (1994, 1997) observed a reliable involvement of the cytoplasm and nucleus × cytoplasm interaction in determining the parameters "total number of embryoids" and "number of green regenerants". Significant nucleus × cytoplasm interactions were observed also by Ekiz and Konzak (1991b, 1991c, 1997), using alloplasmic lines, in which a *T. aestivum* nucleus was transferred to alien cytoplasms by substitution backcrosses.

OBTAINING DIHAPLOIDS

Successful chromosome doubling of microspore-derived haploids is necessary for propagating homozygous plants. Colchicine is the most frequently used chromosome-doubling agent. It blocks mitosis at metaphase by inactivation of the spindle mechanism (Levan 1938; Jensen 1974). In general, the treatments are carried out on whole plants by soaking the crown for several (i.e. 5) hours in colchicine (1 g/l) solution (Mentewab and Sarrafi 1997). Colchicine treatment to plants is rather complicated and can cause high mortality due to the toxic effect of the agent. The conventionally treated plants have low fertility, which could be explained by differences among cells for chromosome doubling in the treated organized tissue. The success of colchicine treatment applied to a plant can depend on many internal and external factors (physiological conditions and deve-

lopmental stage of the plants, environmental conditions, etc.), which places some limitations on the reliable application of the technique. Generally, to obtain a non-chimeric doubled haploid it is necessary to induce doubling at the single-cell stage. Genome doubling should occur preferably before the first microspore mitosis of the microspores in cultured anthers as in this way homozygosity is obtained. Zhuang and Jia (1980) increased the percentage of diploid plants by soaking haploid callus for 72 h in colchicine solution (0.01-0.04%) before transferring them to regeneration medium. Barnabás *et al.* (1991) studied the direct effect of colchicine on the androgenic development of the microspores in *T. aestivum* anthers and compared the effectiveness of the different doubling procedures on the fertility of microspore-derived wheat plants. In their experiment, chromosome doubling due to the direct effect of colchicine on young microspores proved to be effective. Colchicine applied during the induction phase resulted in significantly higher fertility of the regenerated (R_0) plants. Seed production of the plants was improved especially at 0.02 and 0.04% colchicine. The action of colchicine on the cultured microspores was efficient, and the plants (R_0 and R_1) originating from the doubled microspores were fertile. Mentewab and Sarrafi (1997) examined the efficiency of different colchicine treatments on embryoids obtained 45 days after anther culture. A concentration of 500 mg/l of colchicine was used for 1 or 3 days for embryoid treatment. In most cases there were no significant differences between 1 or 3 days treatments, except for 2 out of 5 genotypes tested, which could not regenerate green plants with 3 days treatment. Chromosome doubling rates varied according to genotype. The authors suggest colchicine treatment on embryoids to be tested with 200 to 500 mg/l of colchicine and 1 to 3 days treatment duration to determine the optimal combination. In the study of Ouyang *et al.* (1994) haploid wheat embryoids treated for 1 to 4 days in regeneration media containing colchicine increased chromosome doubling frequency of plants derived from these embryoids. The authors determined that the suitable concentration of colchicine to treat embryoids was around 250 mg/l and the optimal duration was 1 day.

Colchicine treatment is quite effective and less genotype-dependent than other treatments (Mentewab and Sarrafi 1997). This technique has many advantages as it needs less time, labor and space than other techniques. The induction frequency of microspore-derived haploid structures was not greatly influenced by colchicine and this allows for the technique to be used in practical wheat breeding. It is suitable for genotypes with good embryo induction frequencies or low rate of spontaneous plantlet chromosome doubling.

CONCLUSION

Anther culture is one of the most efficient methods for obtaining haploid plants. Besides the fact that by anther culture homozygous lines can be produced within one generation, haploid cell cultures are also useful material for many aspects of plant biotechnology. They provide a number of research opportunities for basic and applied science (Heberle-Bors *et al.* 1996). The male gametophyte is excellent for studying gene regulation, cell fate determination and cellular differentiation in plants. This is due to its relative simplicity compared to the sporophyte, and its accessibility for cytological and molecular analysis (Barinova *et al.* 2002). Discovering the triggers of embryodevelopment in microspore cultures could lead to a greater understanding of the early stages of embryogenesis in plants. Use of molecular techniques for studying gene activity during gametophytic development will help to understand the factors which divert microspore development into an embryogenic pathway. In this regard, restriction fragment length polymorphism (RFLP) may provide a means of identifying DNA sequences which are closely linked to genes responsible for pollen embryogenesis (Powell 1990). Polyhaploid calli and cell suspensions derived from microspores can be used for induced mutation studies with the advantage that mutations tend to be recessive and masked in polyploidy plantlets (Pauls 1996). Use of mutation and selection in haploid cell cultures simplifies breeding for recessive traits and opens new avenues in breeding practice. Thus, *in vitro* androgenesis may become an important supplement to current breeding programs or it may be used as a novel tool.

Although a significant success has been achieved in the study as well as in the practical application of wheat *in vitro* response, a number of important problems still remain unsolved. As it was shown, the anther culture response is controlled by an interaction of nuclear and cytoplasmic genes which is further modified by environment. That is what renders the optimization of culture conditions suitable for all genotypes difficult. A number of factors influencing the androgenic response of wheat genotypes have to be optimized. One way of further increasing the anther culture efficiency proved to be the modification of the induction media. It is important to establish how low medium-pH influences the efficiency of anther culture. At present there is only limited information and few hypotheses on the physiological effect of medium pH on plant tissue culture. The mechanisms causing changes in the developmental pathway of microspores from gametophytic to sporophytic are still largely unexplained. In the long term, much greater emphasis needs to be placed not only on technological advances, but especially on solving fundamental problems associated with the induction process.

Although it has been established that the genetic control of callus induction and embryogenesis is a result mainly of nuclear gene action, the nature of this control has not yet been fully clarified. The molecular events leading to *in vitro* embryogenesis and regeneration are still insufficiently known. It is most important to study and reveal the mechanisms of genetic control of callus induction and embryogenesis as well as the regulation of the inheritance of regeneration ability in callus culture.

The elucidation of the genetic mechanisms controlling susceptibility to *in vitro* culture is also complicated in cytoplasmic genetic systems and by the insufficient knowledge of the importance of the plasmone. Studies until now have demonstrated that it has a comparatively lesser role than nuclear genes. More detailed and extensive investigation on the specific influence of the interaction between different cytoplasms and nuclear genomes in anther culture response is necessary.

A persistent problem related to effectiveness of anther culture is that a great number of albino regenerants are obtained. Although the frequency of albino shoot differentiation is much more related to the donor genotype than to medium components, albinism can be strengthened or weakened depending on a number of environmental factors. In order to fully understand and overcome albinism, more extensive studies are required. New tools aiding the research on this phenomenon and for the identification of possible candidate genes are provided by the application of bulked segregant analysis with ALFP markers and identification of distinct QTLs affecting the tendency for green/ albino plant formation.

The fact that haploid regeneration traits are heritable characteristics and that there is no significant positive or negative relationship between them and yield traits (Moieni and Sarrafi 1998), makes the successful selection of androgenic and/or yield traits in crossing programs possible. By combining anther culture with chromosome engineering techniques, the production of stable dihaploid lines containing alien chromosome carrying agronomically desirable genes can be greatly accelerated.

The need for studying basic processes in plant development in economically important crops require further investigation of the complex phenomenon of *in vitro* androgenesis.

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