FACTORS INFLUENCING THE EFFICIENCY OF WHEAT ANTHER CULTURE

Csaba Lantos and János Pauk (ORCID: 0000-0002-8909-0529)*

Biotechnology Laboratory, Cereal Research Non-profit Ltd., Szeged, Hungary

* Corresponding author, e-mail: janos.pauk@gabonakutato.hu

Running title: *Lantos and Pauk …… Anther culture in wheat*

Received May 20, 2020; revision accepted September XX, 2020

Using *in vitro* androgenesis serves as a unique opportunity to produce doubled haploid (DH) plants in many species. More benefits of this biological phenomenon have kept these methods in the focus of fundamental research and crop breeding for decades. In common wheat (*Triticum aestivum* L.), *in vitro* anther culture is one of the most frequently applied DH plant production methods. The efficiency of *in vitro* wheat anther culture is influenced by many factors, such as the genotype, growing conditions, collection time, pre-treatments, and compositions of media and culture conditions. According to some critical review, the genotype dependency, low efficiency and albinism are mentioned as limitations of application of the anther culture method. However, some research groups have made significant efforts to diminish the effects of these bottlenecks. Due to the improvements, a well-established *in vitro* anther culture method can be an efficient tool in modern wheat breeding programs.

Keywords: androgenesis, anther culture, doubled haploid, *Triticum aestivum* L., wheat

INTRODUCTION

Conventional cross-breeding programs have been dominant in wheat breeding up to now. Modern biotechnological methods seek opportunities to enhance the efficiency of selection and accelerate the breeding process. *In vitro* androgenesis (anther culture and isolated microspore culture) and distant crosses belong to these methods. In anther- and isolated microspore culture, the re-programmed microspores can produce genetically homozygous doubled haploid (DH) plants after spontaneous or induced duplication of the haploid chromosome number. The present review highlights the *in vitro* anther culture from the viewpoint of bread wheat (*Triticum aestivum* L.) breeding.

Anther culture has many advantages for breeding and research programs in crop plants. One of the most important benefits is production of homozygous lines within one generation. The rapid propagation of DH lines can be combined with selection to get inbred lines and accelerate the breeding of new varieties. The DH plant production methods offer an opportunity for quick selection of the recessive alleles. Furthermore, the *in vitro* anther culture can be combined with other value added methods such as marker assisted selection (MAS), QTL analyses, genetic transformation or induced mutation to achieve the desired goals of breeding and research (Chauhan and Khurana, 2011; Wessels and Botes,

2014; Barakat et al., 2017; Ren et al., 2017; Song et al., 2017; Shchukina et al., 2018; Tyrka et al., 2018; Shi et al., 2019; Testillano, 2019; Wajdzik et al., 2019; Bilichak et al., 2020).

At the beginning of wheat *in vitro* haploid research, the anther culture-derived wheat plantlets were published by three research groups in 1973 (Ouyang et al., 1973; Picard and De Buyser, 1973; Wang et al., 1973). After many significant improvements, the first DH variety ('Jinghua No-1') was released by Chinese researchers (Hu et al., 1986), which was followed by some European varieties in a short time, such as 'Florin' (De Buyser et al., 1987) and 'GK Délibáb' (Pauk et al., 1995). In the last two decades, the DH breeding based on anther culture has remained in the focus of some wheat breeding programs, and a number of DH varieties were released and mentioned in scientific publications, for example 'SV Agaton' (Tuvesson et al., 2003), 'McKenzi' (Graf et al., 2003) or 'AC Andrew' (Sadasivaiah et al., 2004), 'Huapei 8' (Ming-hui et al., 2011), 'Kharoba' (Elhaddoury et al., 2012) and 'GK Déva' (Pauk et al., 2020). Moreover, the DH plant production methods can also play a key role in hybrid wheat breeding programs (Longin et al., 2014).

Despite these promising results, several publications informed about bottlenecks (genotype dependency, albinism, low efficiency of green plant production) in connection with anther cultures (Li et al., 2013; Zhao et al., 2015, 2017; Wessels and Botes, 2014; Weigt et al., 2016; Orlowska et al., 2020). However, many efforts and improvements have been made by different research groups to diminish the effects of these factors and enhance the efficiency of green plantlets production in wheat anther culture.

FACTORS INFLUENCING EFFICIENCY OF ANTHER CULTURE

The efficiency of *in vitro* wheat anther culture is influenced by many factors, such as the genotype, growing conditions, collection time, pre-treatments of the donor materials, compositions of induction and regeneration media and culture conditions. The complex of these factors determines the efficiency of *in vitro* anther culture.

GENOTYPE DEPENDENCY

The genotype dependency has been known since the beginning of *in vitro* androgenesis research in wheat. The inheritance of responsibility is determined dominantly by additive effects of the chromosome region (Lazar et al., 1984; Agache et al., 1989; Szakács et al., 1988; Tuvesson et al., 1989), but non-additive effects and cytoplasmic effects were also observed and confirmed in anther culture (Lazar et al., 1984; Agache et al., 1989; Ekiz and Konzak, 1991a, 1991b; Orlov et al., 1999). In the cited articles, many genotypes were already screened for androgenic responses. These experiments were carried out not only on the model genotypes but also on practically important breeding materials.

Some high responsive genotypes (for example 'Chris', 'Pavon', 'Svilena') were identified and have become the favorite test genotypes in *in vitro* androgenesis research (Lazar et al., 1984; Lantos et al., 2013; Castillo et al., 2015; Nielsen et al., 2015; Seifert et al., 2016). Some other genotypes are mentioned as low responsive genotypes ('Berengar', 'Walter', 'Caramba') in anther culture (Torp et al., 2001, Lantos et al., 2013; Castillo et al., 2015). The high- and low responsive genotypes can be used for improvements of well-established protocols to decrease genotype dependency and the effect of genotype×treatment interactions. However, the efficient application of protocols should be proved in modern breeding programs.

Some publications reported the effect of heterosis and additive genes in anther culture of wheat (Lazar et al., 1984; Deaton et al., 1987; Agache et al., 1989; Tuvesson et al., 2000, 2003; Lantos et al., 2019), which can be useful from the viewpoint of practical breeding. The application of responsive genotypes (at least one green plantlet/spike) for crossing can increase the number of produced DH lines in practical breeding programs (Tuvesson et al., 2000, 2003). However, we should get information about

the responsibility of parental lines before the crossing, which can prolong the process of DH breeding. Furthermore, the low responsibility of the desired parental lines can decrease the number of suitable combinations and delay the achievement of the breeding goals. Generally, application of segregating breeding materials can increase the number of DH lines produced in a well-established *in vitro* system (anther- or isolated microspore culture).

Many chromosomes (1A, 1B, 1D, 2D, 5B, 7A, 7B, 7D) and QTLs (1B, 2AL, 2BL, 5BL, 7B) which influence the embryo formation and plant regeneration of *in vitro* anther- and isolated microspore culture have been reported (Szakács et al., 1988; Agache et al., 1989; Kaleikau, 1989; Galiba et al., 1986; Ghaemi et al., 1995; Torp et al., 2001; Nielsen et al., 2015). However, the application of these QTLs have not become widespread in the breeding programs up to now, except for Zhao et al. (2015) who suggested this approach for wheat breeding . In their research and breeding program, they focused on the selection of promising breeding lines with high anther culture ability. Generally, integration of these QTLs in the breeding lines is not the main goal of wheat breeders. Moreover, it can be a risk because of unknown side effects of QTLs in breeding programs.

The methodological improvements remained the best approach to accelerate the breeding process using *in vitro* anther culture. In these experiments, the main goals are the improvement of green plantlets production, reduction of the number of albinos and genotype dependency.

GROWING CONDITIONS

The quality of donor plants is one of the most important critical factors that influence the efficiency of *in vitro* androgenesis in anther culture. The healthy (grown under ideal growing condition) donor plants, tillers and spikes are the first important factors in the implementation of the large-scale DH plant production. Two frequently applied possibilities (a, controlled conditions in a greenhouse and phytotron chamber; b, field conditions in breeding nursery) exist for breeders and researchers in this context.

Controlled light and temperature conditions (greenhouse, phytotron chamber) offer a good chance for growing donor plants during the whole year (Ghaemi et al., 1995; Torp et al., 2001; Pauk et al., 2003; Tuvesson et al., 2000, 2003; Soriano et al., 2007, 2008; Broughton, 2008, 2011; Redha and Suleman, 2011; Brew-Appiah et al., 2013; Sanchez-Diaz et al., 2013; Castillo et al., 2015; Rubtsova et al., 2013; Nielsen et al., 2015; Seifert et al., 2016; Barakat et al., 2017; Sen, 2017; Coelho et al., 2018; Wang et al., 2019; Orlowska et al., 2020; Broughton et al., 2020). The optimized conditions (temperature, light and humidity) can provide healthy donor plants. The winter type genotypes require a vernalization period (6-8 weeks at 3-4°C) after germination. Generally, the donor plants are grown at approximately 18-21/12-15°C (day/night) with 12-18 h photoperiod and 70-80% humidity (Soriano et al., 2007, 2008; Sanchez-Diaz et al., 2013; Castillo et al., 2015; Coelho et al., 2018; Wang et al., 2019; Broughton et al., 2020). Furthermore, the donor plants are regularly nourished with a fertilizer solution. The donor materials grown in controlled conditions can be used for methodological improvements and in applied research programs all the year round.

Field-grown materials are preferred by some research groups (Pauk et al., 2003; Chauhan and Khurana, 2011; Lantos et al., 2013; Zhao et al., 2015, 2017; Weigt et al., 2016, 2019; Lazaridou et al., 2017). Generally, field-grown donor plants produce more tillers with bigger spikes, more anthers and microspores within anthers. Understandably, these facts increase the efficiency of anther culture (green plantlets/100 anthers). Large-scale production of DH lines can be synchronized by using field-grown materials in practical breeding.

COLLECTION TIME OF DONOR PLANTS

The developmental stage of the microspores is one of the most critical factors in the efficient induction of *in vitro* androgenesis. In anther- and isolated microspore culture of wheat, the process of microspore embryogenesis was induced and tracked to study the development, first cell divisions and embryo

formation of microspores (Indrianto et al., 2001; Datta, 2005; Shariatpanahi et al., 2006; Dwivedi et al., 2015; Seldimirova et al., 2017; Niazian and Shariatpanahi, 2020). According to the published protocols of wheat anther culture, *in vitro* androgenesis can be induced in a narrow range of the developmental stages of microspores (uninucleate vacuolated microspores). From this viewpoint, possible differences can be observed among the published methods and protocols. In most publications, the donor tillers were collected when the microspores were at mid- to late uninucleate stages (Soriano et al., 2007, 2008; Broughton, 2008, 2011; Chauhan and Khurana, 2011; Redha and Suleman, 2011; Rubtsova et al., 2013; Sanchez-Diaz et al., 2013; Zhao et al., 2015; Castillo et al., 2015; Echávarri and Cistué, 2016; Weigt et al., 2016, 2019; Lazaridou et al., 2017; Broughton et al., 2020; Orlowska et al., 2020), while other researchers applied early- and mid uninucleate microspores for androgenesis induction in wheat anther culture (Datta and Wenzel, 1987; Pauk et al., 1991, 1995; Tuvesson et al., 2000, 2003; Datta, 2005; Lantos et al., 2013; Lantos and Pauk, 2016). According to our results, the second mentioned alternative is recommended for carrying out an efficient wheat anther culture (Pauk et al., 1991; Pauk et al., 1995; Lantos et al., 2013; Lantos and Pauk, 2016; Kanbar et al., 2020).

PRE-TREATMENTS OF DONOR MATERIALS

Stress pre-treatments play a key role in reprogramming of microspores towards the sporophyte pathway, which practically means induction of *in vitro* embryogenesis. In wheat, researchers have reported many stress factors which were applied for induction of androgenesis, including cold, heat, starvation, colchicine, osmotic shock, 2-HNA, DMSO, etc. (Liu et al., 2001; Barnabás, 2003; Shariatpanahi et al., 2006; Echávarri and Cistué, 2016). However, the most frequently applied stress factors are cold, heat and starvation pre-treatment alone or in combinations. The optimal application and combination of stresses (cold, heat, etc.) are essential in androgenesis induction because too mild or excessive stresses can decrease the efficiency. The increased stresses can reduce the plant regeneration efficiency or enhance the frequency of albino plantlets (Niazian and Shariatpanahi, 2020). The applied stress treatments were implemented on tillers or isolated anthers directly.

Cold pre-treatment of donor tillers offers the easiest way of reprogramming of microspores. *In vitro* androgenesis of microspores can be induced via long cold pre-treatment (2-5°C, 10 days - 4 weeks) of donor tillers (Pauk et al., 2003; Lantos et al., 2013; Lantos and Pauk, 2016; Coelho et al., 2018; Wang et al., 2019).

Short cold pre-treatment (3-8 days, 4-6°C) can also be used for induction of androgenesis (Ghaemi et al., 1995; Broughton, 2008, 2011; Rubtsova et al., 2013; Zhao et al., 2015, 2017; Weigt et al., 2016, 2019; Lazaridou et al., 2017; Sen, 2017). Furthermore, *in vitro* androgenesis was successfully induced also by starvation (alone or in combination with chemical treatments) of isolated anthers (Soriano et al., 2007, 2008; Sanchez-Diaz et al., 2013; Castillo et al., 2015; Echávarri and Cistué, 2016). In anther culture of wheat genotypes, the combined application of mannitol and colchicine increased the number of green plantlets and DH plants depending on the genotype (Soriano et al., 2007), while the combinations of mannitol and DMSO pre-treatment have increased the number of embryoids, green plantlets and DH plants in comparison with starvation applied alone (Echavarri and Cistué, 2016). The pre-treatment of anthers is a time-consuming step, which can require more manual work in *in vitro* plant production.

Heat treatment (3 days, 32 ˚C) of isolated anthers in anther culture is a frequently applied stress factor (Ouyang et al., 1983; Pauk et al., 2003; Shariatpanahi et al., 2006; Lantos et al., 2013; Lantos and Pauk, 2016), which enhances the efficiency of androgenesis induction.

MEDIA COMPOSITION AND CULTURE CONDITION

Many published induction media (AM, C17, P2, P4, LIM, W14, MS3M) can be found in different protocols which were applied successfully in anther culture of wheat. These media were supplemented dominantly with maltose (Hunter, 1987) as a carbon source and Ficoll as an osmotic agent (Datta and

Wenzel, 1987). Combinations of different growth regulators (2,4-D, benzyladenin, centrophenoxine, dicamba, indole-3-acetic acid, kinetin, etc.) were applied in the induction media (Pauk et al., 2003; Soriano et al., 2007; Broughton, 2008; Tuvesson et al., 2000; Ming-hui et al., 2011; Lantos et al., 2013; Rubtsova et al., 2013; Castillo et al., 2015; Weigt et al., 2016, 2019; Zhao et al., 2015, 2017; Orlowska et al., 2020).

Recently, W14 (Ouyang et al., 1989; Lantos et al., 2013; Rubtsova et al., 2013; Lantos and Pauk, 2016; Lazaridou et al., 2017; Zhao et al., 2017) and MS3M media (Soriano, 2007; Sanchez-Diaz et al., 2013; Castillo et al., 2015; Echávarri and Cistué, 2016) have been used frequently in haploid experiments and wheat breeding programs. Modified W14 medium, which has a new name, W14mf synthetic medium, has been applied efficiently in our wheat research and breeding programs (Lantos et al., 2013, 2018, 2019; Lantos and Pauk, 2016; Kanbar et al., 2020; Pauk et al., 2020).

Some organic components (potato extract, wheat ovaries) were reported to enhance the efficiency of *in vitro* anther culture (Chuang et al., 1978; Datta and Wenzel, 1987; Brougton, 2008, 2011; Castillo et al., 2015; Sen, 2017; Broughton et al., 2020). Presumably, the active signal molecules of ovaries like nurse agents support the development of microspore-derived embryoids and plant regeneration (Zur et al., 2015; Niazian and Shariatpanahi, 2020). The ovary conditioned media were reported as an efficient induction medium for plant production in wheat (Soriano et al., 2007, 2008; Broughton, 2011; Sanchez-Diaz, 2013; Castillo et al., 2015; Echávarri and Cistué, 2016). Preparation of these media is labor intensive, so more manual work should be calculated during the process. Furthermore, application of these components implies some effects, such as the genotype and developmental stage of ovaries, which influence the efficiency of the applied method (Castillo et al., 2015). Letarte et al. (2006) made significant efforts to clarify the effect of ovary co-culture, and they proved the positive effect of arabinogalactans and arabinogalactan proteins (AGPs) in induction of *in vitro* androgenesis of wheat (Letarte et al., 2006). The exogenous AGPs has positive influence on the first cell division and development of microspore-derived embryoids (Broughton, 2008; Testillano, 2019), and plant regeneration in anther culture of barley (Makowska et al., 2017). In the improvement of induction media, it would be important to identify new chemicals which play a role in the microspore divisions, the development of multicellular structures and reduction of the stress induced cell death (Testillano, 2019; Weigt et al., 2019; Niazian and Shariatpanahi, 2020). In anther- and isolated microspore culture of wheat, many experiments were implemented to enhance the induction of androgenesis via n-butanol, antioxidants, antibiotics and Trichostatin A (Soriano et al., 2008; Asif et al., 2013a, 2013b; Jiang et al., 2017; Wang et al., 2019; Niazian and Shariatpanahi, 2020). These approaches can open new perspectives for practical application in wheat breeding.

The most frequently applied plant regeneration media are 190-2 (Tuvesson et al., 2000; Pauk et al., 2003; Lantos et al., 2013; Lantos and Pauk, 2016; Lazaridou et al., 2017; Orlowska et al., 2020), J25-8 (Soriano et al., 2007, 2008; Castillo et al., 2015; Echávarri and Cistué, 2016) and MS (Ming-hui et al., 2011; Rubtsova et al., 2013; Zhao et al., 2015, 2017; Weigt et al., 2016, 2019) depending on different research groups. In the plant regeneration period, the embryoids are maintained at 22-26°C with 16 h photoperiod in a growing chamber and they regenerate green and albino plantlets in a different ratio.

ALBINISM IN WHEAT *IN VITRO* ANDROGENESIS

Albinism is a complex phenomenon influenced dominantly by each of the above-mentioned parameters. In the process of *in vitro* androgenesis induction of cereals, albinism is a particular biological phenomenon. Many valuable experiments were carried out to clarify the potential causes of the albinism (Torp et al., 2001; Kumari et al., 2009; Nielsen et al., 2015; Zhao et al., 2017). Different causes which can contribute to the phenomenon of albinism in combination with each other were identified; they include the genotype, growing condition of donor plants, culture conditions, media compositions, incompatibility of nuclear and plastid genomes, deletions or mutations in plastid DNA, up- and down-

regulated genes and proteins, metabolic block in the pathways leading to chlorophyll biosynthesis (Kumari et al., 2009; Nielsen et al., 2015; Zhao et al., 2017; Coelho et al., 2019).

Recently, albinism has been still mentioned as one of the main obstacles of anther culture in wheat *in vitro* androgenesis (Zhao et al., 2015, 2017; Wessels and Botes, 2014; Weigt et al., 2016; Orlowska et al., 2020). From the practical breeding viewpoint, the ratio of albino plantlets can be reduced step by step by optimization of the above mentioned parameters except the genotype of breeding materials, and well-established protocols can be applied efficiently in wheat breeding and research programs (Pauk et al., 2003; Lantos et al., 2013; Castillo et al., 2015; Echávarri and Cistué, 2016; Weigt et al., 2019; Orlowska et al., 2020).

GREEN PLANTLETS PRODUCTION IN WHEAT AS A RESULT OF *IN VITRO* ANDROGENESIS

The genotype dependency still exists in wheat androgenesis, with the green plantlets production ranging from 0 to 325 green plantlets/spike (Castillo et al., 2015; Echavarri and Chistue, 2016; Weigt et al., 2019; Wang et al., 2019; Broughton et al., 2020; Orlowska et al., 2020). Nevertheless, significant progresses have been achieved by application of certain improved protocols resulting in mitigation of the obstacle of genotype dependency (Broughton et al., 2008, 2011, 2020; Soriano et al., 2008; Lantos et al., 2013; Echavarri and Chistue, 2016; Wang et al., 2019; Broughton et al., 2020). The forecast of green plantlets production of wheat breeding materials is difficult due to different methodologies and responsivity of genepools. In the last decade, some studies screened a wide range of breeding materials to check the efficiency of different protocols in practical breeding, and the mean of green plantlets production ranged from 2.00 to 9.76 green plantlets/100 anthers depending on the applied protocols and breeding materials (Lantos et al., 2013, 2016; Weigt et al., 2019, 2020; Kanbar et al., 2020). Recently, the improved methods have been widely applied in wheat breeding programs (Lantos et al., 2013; Echavarri and Chistue, 2016; Wang et al., 2019; Broughton et al., 2020; Pauk et al., 2020).

DOUBLED HAPLOIDS

In *in vitro* plant production methods of crop plants, the number of DH plants is the most important parameter which characterizes the practical application of the methods. In common wheat, most of the applied protocols focused on spontaneous doubling of the haploid chromosome set because of a high percentage (17%-80% depending on genotype) of this phenomenon (Soriano et al., 2007, 2008; Broughton, 2008, 2011; Lantos and Pauk, 2016; Weigt et al., 2019; Broughton, 2020). The number of DH plants can be further increased by *in vivo* or *in vitro* application of colchicine or other chemicals such as amiprophosmethyl, caffeine and triflurain (Barnabás et al., 1991, 2003; Hansen and Andersen, 1998; Pauk et al., 2003; Soriano et al., 2007; Broughton et al., 2020).

CONCLUSION

In the last forty years, many experiments have been carried out to clarify the above mentioned critical points of anther culture (genotype, growing conditions, collection time, etc.) and to diminish the mentioned obstacles (genotype dependency, albinism, limited green plantlets production). Due to these achievements, the anther culture method has become a usable method for wheat breeding and applied research. Recently, the methodological experiments have focused on further improvements of cost efficiency and mitigation of genotype dependency.

The additive components (inducer chemicals, antioxidants, antibiotics, plant growth regulators, polyamines, epigenetic chemicals) of induction medium can play a key role in the enhancement of *in vitro* androgenesis via increasing the frequency of cell divisions, embryo formation, and reduction of the stress-induced cell death. Furthermore, *in vivo* and *in vitro* application of antimitotic agents can increase the number of DH plants, which is the most important parameter from a practical viewpoint of breeding

and applied research. These approaches can open new perspectives to improve genotype independent methods for wheat research and wheat breeding.

AUTHORS' CONTRIBUTIONS

Both authors (CL, JP) contributed equally to this review. The authors declare that they have no conflict of interest.

ACKNOWLEDGMENTS

This project was supported by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences. The authors thank for the support of scientific programs: National Research, Development and Innovation Office (grant number "OTKA-K_16-K119835" and "GINOP-2.2.1-15-2016-00026") and Ministry for Innovation and Technology ("TUDFO/51757/2019-ITM"), and the project tender: 2020-4.1.1-TKP2020 entitled "Combination of wheat biotic and abiotic stress tolerance with yield and quality to produce new profitable wheat varieties with yield stability". Authors thank Elizabeth Búza and Kornélia Hajdú Búza for language revision of the article.

REFERENCES

ASIF M, EUDES F, GOYAL A, AMUNDSEN E, RANDHAWA H, and SPANER D. (2013a). Organelle antioxidants improve microspore embryogenesis in wheat and triticale. *In Vitro Cellular and Developmental Biology - Plant* 49: 489–497.

ASIF M, EUDES F, RANDHAWA H, AMUNDSEN E, YANKE J, and SPANER D. (2013b). Cefotaxime prevents microbial contamination and improves microspore embryogenesis in wheat and triticale. *Plant Cell Reports* 32: 1637–1646.

AGACHE S, BECHELIER B, DE BUYSER J, HENRY Y, and SNAPE J. 1989. Genetic analyses of anther culture response in wheat using aneuploid, chromosome substitution and translocation lines. *Theoretical and Applied Genetics* 77: 7-11.

BILICHAK A, SASTRY-DENT L, SRIRAM S, SIMPSON M, SAMUEL P, WEBB S, JIANG F, and EUDES F. 2020. Genome editing in wheat microspores and haploid embryos mediated by delivery of ZFN proteins and cell-penetrating peptide complexes. *Plant Biotechnology Journal* 18: 1307-1316.

BARAKAT MN, AL-DOSS AA, GHAZY AI, MOUSTAFA KA, ELSHAFEI AA, and AHMED EI. 2017. Doubled haploid wheat lines with high molecular weight glutenin alleles derived from microspore culture. *New Zealand Journal of Crop and Horticultural Science* 46: 198-211.

BARNABÁS B, PFAHLER PL, and KOVÁCS G. 1991. Direct effect of colchicine on the microspore embryogenesis to produce dihaploid plants in wheat (*Triticum aestivum* L.). *Theoretical and Applied Genetics* 81: 675-678.

BARNABÁS B. 2003. Protocol for producing doubled haploid plants from anther culture of wheat (*Triticum aestivum* L.). in: Maluszynski M, Kasha KJ, Forster BP and Szarejko I (eds), Doubled Haploid Production in Crop Plants, a manual, 65-70. Kluwer Academic Publishers, Dordrecht, The Netherlands.

BREW-APPIAH RAT, ANKRAH N, LIU W, KONZAK CF, VON WETTSTEIN D, and RUSTGI S. 2013. Generation of doubled haploid transgenic wheat lines by microspore transformation. *PLOS ONE* 8: e80155.

BROUGHTON S. 2008. Ovary co-culture improves embryo and green plantlets production in anther culture of Australian spring wheat (*Triticum aestivum* L.). *Plant Cell, Tissue and Organ Culture* 95: 185-195.

BROUGHTON S. 2011. The application of n-butanol improves embryo and green plant production in anther culture of Australian wheat (*Triticum aestivum* L.) genotypes. *Crop and Pasture Science* 62: 813-822.

BROUGHTON S, CASTELLO M, LIU L, KILLEN J, HEPWORTH A, and O'LEARLY R. 2020. The effect of Caffeine and Triflurain on chromosome doubling in wheat anther culture. *Plants* 9: 105.

CASTILLO AM, SANCHEZ-DIAZ RA, and VALLES MP. 2015. Effect of ovary induction on bread wheat anther culture: ovary genotype and developmental stage, and candidate gene association. *Frontiers in Plant Science* 6: 402.

CHAUHAN H, and KHURANA P. 2011. Use of haploid technology for development of stable drought tolerant bread wheat (*Triticum aestivum* L.) transgenics. *Plant Biotechnology Journal* 9: 408-417.

CHUANG CC, OUYANG TW, CHIA H, CHOU SM, and CHING CK. 1978. A set of potato media for wheat anther culture. in: *Proceedings of Symposium on Plant Tissue Culture*. Science Press, Beijing, China. p. 51-56.

COELHO MB, SCAGLIUSI SMM, LIMA MIPM, CONSOLI L, and GRANDO MF. 2018. Androgenic response of wheat genotypes resistant to fusariosis. *Pesquisa Agropecuaria Brasileira* 53: 575-582.

DATTA SK, and WENZEL G. 1987. Isolated microspore derived plant formation via embryogenesis in Triticum aestivum L. *Plant Science* 48: 49-54.

DATTA SK. 2005. Androgenic haploids: factors controlling development and its application in crop improvement. *Current Science* 89: 1870-1878.

DEATON WR, METZ SG, ARMSTRONG TA, and MASCIA PN. 1987. Genetic-analyses of the anther culture response of 3 spring wheat crosses. *Theoretical and Applied Genetics* 74: 334-338.

DWIVEDI SL, BRITT AB, TRIPATHI L, SHARMA S, UPADHYAYA HD, and ORTIZ R. 2015. Haploids: Constraints and opportunities in plant breeding. *Biotechnology Advances* 33: 812-829.

DE BUYSER J, HENRY Y, LONNET P, HERZOG R, and HESPEL A. 1987 Florin - a doubled haploid wheat variety developed by the anther culture method. *Plant Breeding* 98: 53-56.

ECHÁVARRI B, and CISTUÉ L. 2016. Enhancement in androgenesis efficiency in barley (*Hordeum vulgare* L.) and bread wheat (*Triticum aestivum* L.) by the addition of dimethyl sulfoxide to the mannitol pretreatment medium. *Plant Cell, Tissue and Organ Culture* 125: 11-22.

EKIZ H, and KONZAK CF. 1991a. Nuclear and cytoplasmic control of anther culture response in wheat: I. analyses of alloplasmic lines. *Crop Science* 31: 1421-1427.

EKIZ H, and KONZAK CF. 1991b. Nuclear and cytoplasmic control of anther culture response in wheat: III. Common wheat crosses. *Crop Science* 31: 1432-1436.

ELHADDOURY J, LHALOUI S, UDUPA SM, MOATISSIM B, TAIQ R, RABEH M, KAMLAOUI M, and HAMMADI M. 2012. Registration of 'Kharoba': A bred wheat cultivar developed through doubled haploid breeding. *Journal of Plant Registrations* 6: 169-173.

GALIBA G, KOVÁCS G, and SUTKA J. 1986. Substitution analyses of plant regeneration from callus culture in wheat. *Plant Breeding* 263: 263-266.

GHAEMI, M, SARRAFI A, and MORRIS R. 1995. Reciprocal substitutions analyses of embryo induction and plant regeneration from anther culture in wheat (*Triticum aestivum* L.). *Genome* 38: 158-165.

GRAF RJ, HUCL P, ORSHINSKY BR, and KARTHA KK. 2003. "McKenzi" hard red spring wheat. *Canadian Journal of Plant Science* 83: 565–569.

HANSEN NJP, and ANDERSEN SB. 1998. Efficient production of doubled haploid wheat plants by *in vitro* treatment of microspores with trifluralin or APM. *Plant Breeding* 117: 401-405.

HU DF, YUAN ZD, TANG YL, and LIU JP. 1986. "Jinghua No-1" a winter-wheat variety derived from pollen sporophyte. *Scientia Sinica Series B, Chemical, Biological, Agricultural, Medical and Earth Sciences* 29: 733–745.

HUNTER CP. 1987. Plant regeneration method. Europen patent, 1987. Application No. 872007737.

INDRIANTO A, BARINOVA I, TOURAEV A, and HEBERLE-BORS E. 2001. Tracking individual wheat microspores in vitro: identification of embryogenic microspores and body axis formation in the embryo. *Planta* 212: 163-174.

JIANG F, RYABOVA D, DIEDHIOU J, HUCL P, RANDHAWA H, MARILLIA EF, FOROUD NA, EUDES F, and KATHIRIA P. 2017. Trichostatin A increases embryo and green plant regeneration in wheat. *Plant Cell Reports* 36: 1701-1706.

KALEIKAU EK, SEARS RG, and GILL BS. 1989. Monosomic analyses of tissue culture response in wheat (*Triticum aestivum* L.). *Theoretical and Applied Genetics* 78: 625-632.

KANBAR OZ, LANTOS C, KISS E, and PAUK J. 2020. Androgenic responses of winter wheat combinations in in vitro anther culture. *Genetika-Belgrade* 52: 337-350.

KUMARI M, CLARKE HJ, SMALL I, and SIDDIQUE KHM. 2009. Albinism in Plants: A major bottleneck in wide hybridization, androgenesis, and doubled haploid culture. *Critical Reviews in Plant Sciences* 28: 393-409.

LANTOS C, BÓNA L, NAGY É, BÉKÉS F, and PAUK J. 2018. Induction of in vitro androgenesis in anther and isolated microspore culture of different spelt wheat (*Triticum spelta* L.) genotypes. *Plant Cell, Tissue and Organ Culture* 133: 385-393.

LANTOS C, and PAUK J. 2016. Anther culture as an effective tool in winter wheat (*Triticum aestivum* L.) breeding. *Russian Journal of Genetics* 52: 794-801.

LANTOS C, PURGEL S, ÁCS K, LANGÓ B, BÓNA L, BODA K, BÉKÉS F, and PAUK J. 2019. Utilization of in vitro anther culture in spelt wheat breeding. *Plants* 8, 436.

LANTOS C, WEYEN J, ORSINI JM, GNAD H, SCHLIETER B, LEIN V, KONTOWSKI S, JACOBI A, MIHÁLY R, BROUGHTON S, and PAUK J. 2013. Efficient application of in vitro anther culture for different European winter wheat (*Triticum aestivum* L.) breeding programs. *Plant Breeding* 132: 149-154.

LAZAR MD, BAENZIGER PS, and SCHAEFFER GW. 1984. Combining abilities and heritability of callus formation and plantlet regeneration in wheat (*Triticum aestivum* L.) anther cultures. *Theoretical and Applied Genetics* 68: 131-134.

LAZARIDOU TB, PANKOU CI, XYNIAS IN, and ROUPAKIAS DG. 2017. Effects of 1BL.1RS wheat-rye translocation on the androgenic response in spring bread wheat. *Cytology and Genetics* 51: 485-490.

LETARTE J, SIMION E, MINER M, and KASHA KJ. 2006. Arabinogalactans and arabinogalactan-proteins induce embryogenesis in wheat (*Triticum aestivum* L.) microspore culture. *Plant Cell Reports* 24: 691–698.

LI H, SINGH RP, BRAUN HJ, PFEIFFER WH, and WANG J. 2013. Doubled haploids versus conventional breeding in CIMMYT wheat breeding programs. *Crop Science* 53: 74-83.

LIU W, ZHENG MY, and KONZAK CF. 2001. Improving green plant production via isolated microspore culture in bread wheat (*Triticum aestivum L.*). *Plant Cell Reports* 20: 821-824.

LONGIN CFH, MI X, MELCHINGER AE, REIF JC, and WÜRSCHUM T. 2014. Optimum allocation of test resources and comparison of breeding strategies for hybrid wheat. *Theoretical and Applied Genetics* 127, 2117-2126.

MAKOWSKA K, KALUZNIAK M, OLESZCZUK S, ZIMNY J, CZAPLICKI A, and KONIECZNY R. 2017. Arabinogalactan proteins improve plant regeneration in barley (*Hordeum vulgare* L.) anther culture. *Plant Cell Tissue and Organ Culture* 131: 247-257.

MING-HUI K, YAN H, BING-YAN H, YONG-YING Z, SHI-JIE W, LI-JUAN M, and XIN-YOU Z. 2011. Breeding of newly licensed wheat variety Huapei 8 and improved breeding strategy by anther culture. *African Journal of Biotechnology* 10: 19701-19706.

NIAZIAN M, and SHARIATPANAHI ME. 2020. In vitro-based doubled haploid production: recent improvements. *Euphytica* 216: 69.

NIELSEN NH, ANDERSEN SU, STOUGAARD J, JENSEN A, BACKES G, and JAHOOR A. 2015. Chromosomal regions associated with the *in vitro* culture response of wheat (*Triticum aestivum* L.) microspores. *Plant Breeding* 134: 255-263.

OUYANG JW, HU H, CHUANG CC, and TSENG CC. 1973. Induction of pollen plants from anthers of *Triticum aestivum* L. cultured *in vitro*. *Scientia Sinica* 16: 79–95.

OUYANG JW, JIA SE, ZHANG C, CHEN X, and FEN G. 1989. A new synthetic medium (W14) for wheat anther culture. Annual Report, 91-92, Institute of Genetics, Academia Sinica Beijing.

OUYANG JW, ZHOU SM, and JIA SE. 1983. The response of anther culture to culture temperature in *Triticum aestivum. Theoretical and Applied Genetics* 66: 101-109.

ORLOV PA, BECKER D, SHEWE G, and LORZ H. 1999. Cytoplasmic effects on pollen embryogenesis induction in wheat microspore culture. *Cereal Research Communications* 27: 357-363.

ORLOWSKA R, PACHOTA KA, MACHCZINSKA J, NIEDZIELA A, MAKOWSKA K, ZIMNY J, and BEDNAREK PT. 2020. Improvement of anther cultures conditions using the Taguchi method in three cereal crops. *Electronic Journal of Biotechnology* 43: 8-15.

PAUK J, KERTÉSZ Z, BEKE B, BÓNA L, CSŐSZ M, and MATUZ J. 1995. New winter wheat variety-"GK Délibáb" developed via combining conventional breeding and *in vitro* androgenesis. *Cereal Research Communications* 23: 251–256.

PAUK J, MANNINEN O, MATTILA I, SALO Y, and PULLI S. 1991. Androgenesis in hexaploid spring wheat F2 populations and their parents using a multiple-step regeneration system. *Plant Breeding* 107: 18-27.

PAUK J, MIHÁLY R, and PUOLIMATKA M. 2003. Protocol of wheat (*Triticum aestivum* L.) anther culture. in: Maluszynski M, Kasha KJ, Forster BP and Szarejko I (eds), *Doubled Haploid Production in Crop Plants, a manual*, 59-64. Kluwer Academic Publishers, Dordrecht, The Netherlands.

PAUK J, LANTOS C, CSEUZ L, PAPP M, ÓVÁRI J, BEKE B, and PUGRIS T. 2020. 'GK Déva' dihaploid módszer segítségével előállított új őszi búzafajta ('GK Déva', new released winter wheat variety using dihaploid method). XXVI. Növénynemesítési Tudományos Napok, Szeged, Hungary, 04-05. 03. 2020. p. 102.

PICARD E, and DE BUYSER J. 1973. Obtention de plantlets haploides de Triticum aestivum L. á partir de cultures d'anthéres in vitro. *Comptes Rendus de l'Académie des Sciences Paris* 277: 1463-1466.

REDHA A, and SULEMAN P. 2011. Effects of exogenous application of polyamines on wheat anther culture. *Plant Cell, Tissue and Organ Culture* 105: 345-353.

RUBTSOVA M, GNAD H, MELZER M, WEYEN J, and GILS M. 2013. The auxins centrophenoxine and 2,4-D differ in their effects on non-directly induced chromosome doubling in anther culture of wheat (*T. aestivum* L.). *Plant Biotechnology Reports* 7: 247-255.

REN J, WU P, TRAMPE B, TIAN X, LÜBBERSTEDT T, and CHEN S. 2017. Novel technologies in doubled haploid line development. *Plant Biotechnology Journal* 15: 1361-1370.

SADASIVAIAH RS, PERKOVIC SM, PEARSON C, POSTMAN B, and BERES BL. 2004. Registration of "AC Andrew" wheat. *Crop Science* 44: 696–697.

SANCHEZ-DIAZ RA, CASTILLO AM, and VALLES MP. 2013. Microspore embryogenesis in wheat: new marker genes for early, middle and late stages of embryo development. *Plant Reproduction*. 26: 287-296.

SEIFERT F, BÖSSOW S, KUMLEHN J, GNAD H, and SCHOLTEN S. 2016. Analyses of wheat microspore embryogenesis induction by transcriptome and small RNA sequencing using the highly responsive cultivar "Svilena". *BMC Plant Biology* 16: 97.

SELDIMIROVA OA, KRUGLOVA NN, TITOVA GE, and BATYGINA TB. 2017. Comparative ultrastructural analyses of the in vitro microspore embryoids and in vivo zygotic embryos of wheat as a basis for understanding of cytophysiological aspects of their development. *Russian Journal of Developmental Biology* 48: 185-197.

SEN A. 2017. Retrotransposon insertion variations in doubled haploid bread wheat mutants. *Plant Growth Regulation* 81: 325-333.

SHARIATPANAHI ME, BAL U, HEBERLE-BORS E, and TOURAEV A. 2006. Stresses applied for the re-programming of plant mmicrospores towards in vitro embryogenesis. *Physiologia Plantarum* 127: 519-534.

SHCHUKINA LV, PSHENICHNIKOVA T, KHLESTKINA EK, MISHEVA S, KARTSEVA T, ABUGALIEVA A, and BORNER A. 2018. Chromosomal Location and Mapping of Quantitative Trait Locus Determining Technological Parameters of Grain and Flour in Strong-flour Bread wheat cultivar Saratovskaya 29. *Cereal Research Communications* 46: 628-638.

SHI YG, LIAN Y, SHI HW, WANG SG, FAN H, SUN DZ, and JING RL. 2019. Dynamic analysis of QTLs for green leaf area duration and green leaf number of main stem in wheat. *Cereal Research Communications* 47: 250-263.

SONG J, CARVER BF, POWERS C YAN L, KLAPSTE J, EL-KASSABY YA, and CHEN C. 2017. Practical application of genomic selection in a doubled-haploid winter wheat breeding program. *Molecular Breeding* 37: 117.

SORIANO M, CISTUÉ L, VALLES MP, and CASTILLO AM. 2007. Effects of colchicine on anther and microspore culture of bread wheat (*Triticum aestivum* L.). *Plant Cell, Tissue and Organ Culture* 91: 225-234.

SORIANO M, CISTUÉ L, and CASTILLO AM. 2008. Enhanced induction of microspore embryogenesis after n-butanol treatment in wheat (*Triticum aestivum* L.) anther culture. *Plant Cell Reports* 27: 805-811.

SZAKÁCS É, KOVÁCS G, PAUK J, and BARNABÁS B. 1988. Substitution analyses of callus induction and plant regeneration from anther culture in wheat (*Triticum aestivum* L.). *Plant Cell Reports* 7: 127-129.

TESTILLANO PS. 2019. Microspore embryogenesis: targeting the determinant factors of stress-induced cell reprogramming for crop improvement. *Journal of Experimental Botany* 70: 2965-2978.

TORP AM, HANSEN AL, and ANDERSEN SB. 2001. Chromosomal regions associated with green plant regeneration in wheat (*Triticum aestivum* L.) anther culture. *Euphytica* 119: 377-387.

TUVESSON S, LJUNGBERG A, JOHASSON N, KARLSSON KE, SUIJS LW, and POSSET JP. 2000. Large-scale production of wheat and triticale double haploid through the use of a single-anther culture method. *Plant Breeding* 119: 455-459.

TUVESSON IKD, PETERSON S, and ANDERSON SB. 1989. Nuclear genes affecting albinism in wheat (*Triticum aestivum* L.) anther culture. *Theoretical and Applied Genetics* 78: 879-961.

TUVESSON S, VON POST R, and LJUNGBERG A. 2003. Wheat anther culture. in: Maluszynski M., Kasha, K.J., Forster, B.P., Szarejko, I. (eds), *Doubled Haploid Production in Crop Plants, a manual*, 71-76. Kluwer Academic Publishers, Dordrecht, The Netherlands.

TYRKA M, OLESZCZUK S, RABIZA-SWIDER J, WOS H, WEDZONY M, ZIMNY J, PONITKA A, SLUSARKIEWICZ-JARZINA A, METZGER RJ, BAENZIEGER PS, and LUKASZEWSKI AJ. 2018. Populations of doubled haploids for genetic mapping in hexaploid winter triticale. *Molecular Breeding* 38: 46.

WAJDZIK K, GOLEBIOWSKA G, DYDA M, GAWRONSKA K, RAPACZ M, and WEDZONY M. 2019. The QTL mapping of the important breeding traits in winter triticale (*X Triticosecale* Wittm.). *Cereal Research Communications* 47: 395-408.

WANG CC, CHU CC, SUN CS, WU HS, YIN KC, and HSU C. 1973. The androgenesis in wheat (Triticum aestivum L.) anthers cultured *in vitro*. *Acta Genetica Sinica* 16: 218–222.

WANG HM, ENNS JL, BROST JM, ORR TD, and FERRIE AMR. 2019. Improving the efficiency of wheat microspore culture evaluation of pretreatments, gradients, and epigenetic chemicals. *Plant Cell Tissue and Organ Culture* 139: 589-599:

WEIGT D, KIEL A, NAWRACALA J, PLUTA M, and LACKA A. 2016. Solid-stemmed spring wheat cultivars give better androgenic response than hollow-stemmed cultivars in anther culture. *In Vitro Cellular and Developmental Biology - Plant* 52: 619-625.

WEIGT D, NIEMANN J, SIATKOWSKI I, ZYPRYCH-WALCZAK J, PRZEMYSLAW O, and KURASIAK-POPOWSKA D. 2019. Effect of zearalenone and hormone regulators on microspore embryogenesis in anther culture of wheat. *Plants* 8: 487.

WEIGT D, KIEL A, SIATKOWSKI I, ZYPRYCH-WALCZAK J, TOMKOWIAK A, and KWIATEK M. 2020. Comparison of androgenic response of spring and winter wheat. *Plants* 9: 49.

WESSELS E, and BOTES WC. 2014. Accelerating resistance breeding in wheat by integrating marker-assisted selection and doubled haploid technology. *South. African Journal of Plant and Soil* 31: 35-43.

ZHAO L, LIU L, WANG J, GUO H, GU J, ZHAO S, LI J, and XIE Y. 2015. Development of a new wheat germplasm with high anther culture ability by using of gamma-ray irradiation and anther culture. *Journal of the Science of Food and Agriculture* 95: 120-125.

ZHAO P, WANG K, ZHANG W, LIU HY, DU LP, HU HR, and YE XG. 2017. Comprehensive analyses of differently expressed genes and proteins in albino and green plantlets from a wheat anther culture. *Biologia Plantarum* 61: 255-265.

ZUR I, DUBAS E, KRZEWSKA M, and JANOWIAK F. 2015. Current insight into hormonal regulation of microspore embryogenesis. *Frontiers in Plant Sciences* 6: 424.