NARDI FUNDULEA, ROMANIA
www.incda-fundulea.ro

ROMANIAN AGRICULTURAL RESEARCH, NO. 32, 2015
Print ISSN 1222-4227; Online ISSN 2067-5720

IN VITRO RESPONSE OF ANTHERS FROM DIFFERENT BARLEY GENOTYPES IN ORDER TO OBTAIN HAPLOID PLANTS

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ABSTRACT

A comparative study of anthers response to *in vitro* culture from 5 barley (*Hordeum vulgare* L.) genotypes was carried out. For androgenesis induction we used 8 pretreatment schemes and 3 initiation nutrient media. Cytological evaluation of anther derived-structure revealed the formation of calli and embryogenic structures, microspore embryogenesis providing a complex morphogenetic pathway, not only dependent on the studied factors, influenced dedifferentiation. On base of histological evaluation a set of restructuring were described, which conducted to embryo differentiation from globular stage; pre-embryonic structures to embryo formation. These reorganizations may be inhibited and stopped at any development stage. The androgenic capacity was found related to accumulation of storage of substances and phenolic compounds. Evaluation of morphological and structural features of morphogenetic structures initiated from barley anthers, proved the complexity of androgenetic process, dependent on the stage of generating microspore dedifferentiation, including pretreatment conditions and the genotype peculiarities.

Key words: androgenesis, microspore, *in vitro* culture, barley, callus, embryogenesis.

INTRODUCTION

Microspores or young pollen grains can be switched from their normal pollen development towards an embryogenic pathway, a process called androgenesis. Five major ways that support the embryo development have been identified. Multinucleate structures can be generated by division of the uninucleate microspore or in young pollen grains by division of the vegetative cell and/or generative cell. In some plants, nuclear fusion between the vegetative cell and the generative cell prior to division, as well as the initial formation of a syncytium have also been described (Soriano et al., 2013). Anther culture has been shown to require earlier stages of microspore development than isolated microspore culture (Hoekstra et al., 1992). Anther tissues could provide a better environment, in which microspores at early stages can develop, by providing nutrients and protection against stress. The anther wall has been proposed for isolating the microspores from the culture medium. The delay in the timing of induction necessitates the use of earlier stages as starting material (Salas et al., 2012). The reprogramming of the anther tissues and microspore developmental can be induced by application of a stress treatment, usually temperature, nutrient or osmotic stress, either alone or in combination (Islam and Tuteja, 2012; Shariatpanahi et al., 2006). The cold treatment of the spikes is mostly used (Huang and Sunderland, 1982); immersing in a mannitol solution is also a widespread procedure (Roberts-Oehschlager and Dunwell, 1990). Kruczkowska et al. (2002) demonstrated the efficacy of pretreatment at low temperature (4C° during 28 days) in callus induction and embryogenesis. It was established that the low temperatures caused a lack of synchronization between tapetum development and microspores (Powell, 1988), while the mannitol intensifies osmotic pressure and nutrient transport (Hoekstra et al., 1997). Reprogramming of cellular metabolism includes the repression of gene expression related to starch biosynthesis and the induction of proteolytic genes (e.g. components of the 26S proteasome, metalloprotease, cysteine, and aspartic proteases) and stress-related proteins (Maraschin et al., 2005). It has been

Received 12 June 2014; accepted 16 March 2015. First Online: April, 2015. DII 2067-5720 RAR 2015-110

established, that cold stress causes reorientation in the spindle of I division resulting in formation of microspores with two equal nuclei (D'yachuk et al., 2010). Low temperatures delay the destruction of anther cell wall, which causes an adverse impact on development of isolated microspores in culture, and, at the same time, cold prolongs viability of microspores, as well as the period suitable for introduction of the anthers to *in vitro* culture. Histological aspects preceding sporophytic cells derived from anther remain unclear.

The overall goal of the present study was to identify the structural reorganization of embryogenic and non-embryogenic cell cultures derived from barley anthers.

MATERIAL AND METHODS

The spring (Galactic, Unirea, Sonor) and winter (Stralucitor) barley cultivars and the variety *hexastichum* served as biological material.

Donor plants were grown in controlled conditions, in optimal conditions concerning photoperiod, light intensity, temperature and nutrition, according standards recommended by Cistue et al. (2003). Spikes were collected when most of the microspores were at the mid to late-uninucleate stage. For cytological identification of meiosis stages the anthers were fixed in Carnoy solution (3:1) and stained with aceto-carmine. Collected spikes were rinsed in water with three drops of Tween-20 (0.1%) and under running tap water for 10 min; after that the spikes (60-80 mm long) were sterilized with 70% ethylic alcohol followed by 5.2 % sodium hypochlorite (dilution with distillate water 1:1). At last, in laminar airflow hood the anthers were excised and incubated in culture medium.

To establish an efficient protocol for barley genotypes with local agronomic interest we used 8 schemes of pretreatment:

No 1 – T=4°C, Mannitol 0.7M, followed by the passage supplemented with 0.34M Mannitol;

No 2 – T=4°C, Mannitol 0.34M, followed by the passage supplemented with 0.17M Mannitol;

No 3 – T=4°C, Mannitol 0.34M;

No 4 – Mannitol 0.34M;

No 5 – T=4°C, during 10-14 days;

No 6 – T=4°C, Mannitol 0.34M+H_2O d;

No 7 – T=4°C, H_2O d, during 10-14 days;

No 8 – Mannitol – 0.17M;

No 9 – control, lots without pretreatment.

As induction medium we used three variants: C_3 (Jacquard et al., 2003), N_6 (Chu, 1978) and FHG (Hunter, 1988). For each variant in medium 25-30 anthers were inoculated in Petri dishes and incubated at 25±2°C in dark for 14 days.

After 30 days the explants were transferred to dishes with fresh medium and exposed to a constant temperature 25±2°C, with 16 hours photoperiods and light 2000lux. Every 30 days we performed passages, respecting the aseptic rules.

Cytological examination of semi-thin sections, using light microscopy techniques, was performed to identify the structural peculiarities of embryogenic and non-embryogenic cells population derived under *in vitro* anthers culture. Samples were fixed with 4% aqueous glutaraldehyde and post-fixed with 1% osmium tetroxide. After dehydration in ascending ethanol concentrations and propylene oxide the material was embedded in a mixture of Epon. Semi-thin sections, produced on ultramicrotome UMTP 3, were stained with methylene blue.

The statistical processing of data was carried out using the software package Statgraphics Plus for Windows (version 2.1; Microsoft Corp., Redmond, WA, USA) and Microsoft Excel. The contribution of variation sources was computed following the ANOVA test (Clewer and Scarisbrick, 2001).

RESULTS AND DISCUSSION

According to the literature, the final stage of the second meiotic division that leads to the formation of unicellular microspores is considered the most favorable for the induction of androgenesis. For barley, the same as for other monocotyledonous plants, the successive type of microspores formation

is specific. Also, there is a heterogeneity of the course of meiosis in the same anther, so on the same sample pollen mother cells in phases I and II of meiosis (from metaphase I to telophase II and tetrade stage) were attested. Cytological studies revealed the unicellular microspore predominance in anthers with a length of about 6-7 cm for varieties Galactic, Sonor and Unirea, compared to 7-8 cm for barley cultivar Stralucitor.

During *in vitro* cultivation of barley anthers on initiation medium, the majority presented no reaction, microspores showing vacuolization of vegetative cell, developing further the death program with further degeneration (Figure 1). Only a very small amount of anthers passed to androgenetic pathway.

Following the analysis of semi-thin sections we observed sterile microspores, assisted by the compact anther wall, e.g. endothecium was represented by 2-3 rows of cells (Figure 1A). The anthers with androgenic response contained fertile microspores with content-rich intracellular structures (Figure 1B). Microspores sizes varied within same pollen sac. As result of dedifferentiation inside microspore, embryonic structures were generated (Figure 1D), accompanied further by the exine break and destruction of sterile anther tissues (endothecium and tapetum).

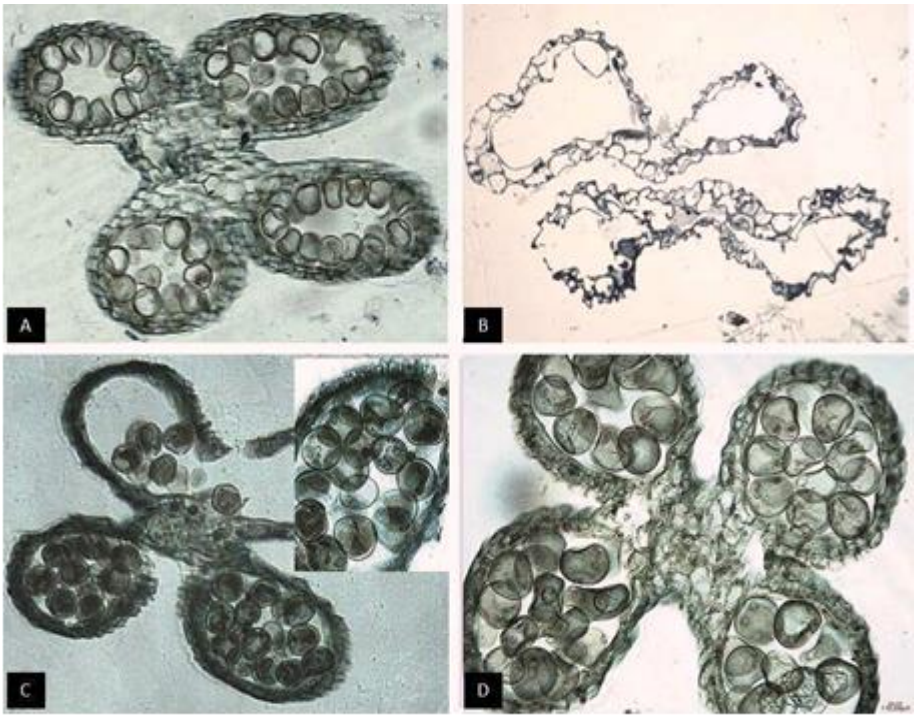

Figure 1. Semi-thin section through barley anthers with different reaction to pretreatment: anthers without response (A), necrotic anthers (B), cold pretreatment of harvested spikes at T=4°C in Mannitol 0.34M, followed by the passage supplemented with 0.17M Mannitol (pretreatment scheme No 2) (C), cold pretreatment of harvested spikes at T=4°C, during 10-14 days (pretreatment scheme No 5) (D)

Based on conducted research we found that the essential role in the induction of androgenetic response is played by genotype. Despite using different pretreatment schemes involving various stress factors, it was not possible to initiate a positive reaction for variety *hexastichum.* The formation of various androgenetic structures (embryoids and calli) was observed after 3 weeks of culture for the anthers exposed to the mannitol starvation at cold pretreatment (T=4°C) for 3-4 days (cv. Galactic) and 14 days (cv. Sonor, Unirea) (Table 1).

Table 1. Anthers reaction to pretreatment procedures and culture medium

Genotype	Sp.No	Pretreatment																	
		No 1		No 2		No 3			No 4		No 5			No 6		No7	No8	No9	
		C_3	FHG	N_6	C_3	N_6	C_3	FHG	N_6	C_3	N_6	C_3	FHG	N_6	C_3	FHG	N_6	C_3, N_6, FHG	
Sonor	17																		
Unirea	20																		
Galactic	13																		
Straluci-tor	11																		

Note: – calli, – embriogenic structure, – albino regenerants, – green regenerants, – necrosis, – anthers without responses.

According to the literature data, for the induction dedifferentiation and reclaimed sporophytic development the importance of nutrient medium and hormone balances has been demonstrated.

Of the 16 experimental variants derived from pretreatment schemes and three nutrient media a stimulatory effect in inducing androgenetic responses, depending on the genotype was established.

Anthers with positive reaction during further cultivation showed a different degree of callus induction or embryogenic structure formation (Figure 2).

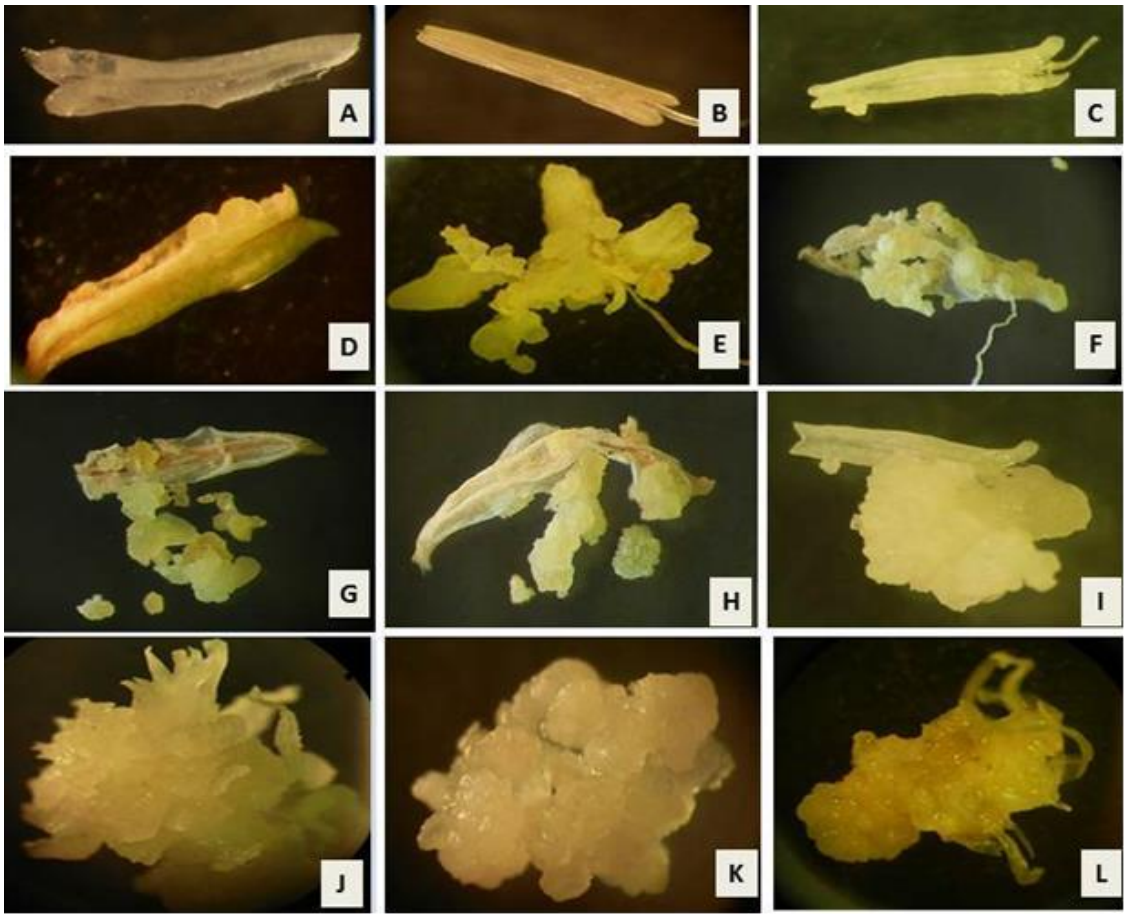

Figure 2. Morphological aspect of anthers and derived structure under *in vitro* cultivation: necrotic anther (A), anther without response (B), initiation of callus induction (C), anther with different potential of callus induction (D-I, K), embryogenic structure differentiation (J, L)

The intensity of cell proliferation was found different. The morphology of derived cell mass: also varied from fluid to dense – fragile or compact structures.

Subsequent development processes and further cell proliferation was also dependent on conditions, nutritive medium composition and genotype. FHG was found favourable for ensuring development. This phase is quite important in determining the success of the whole process of androgenesis, or directing to the browning and cells necrosing. Senescence processes are marked by morphological changes, which developed from the periphery to the centre more brittle and mate. It showed a slight mass loss, determined by callus cell dehydration.

According to the same authors the callus formation may derive from somatic tissues of cultured anthers (Bal et al., 2012; Chen et al., 2005; Gribaudo et al., 2004). That is considered as a rather common phenomenon reported in many species and it remains a source of anther-derived plants. Some of the anther wall cells, e.g. endothecium of connective tissue cells, dedifferentiate and enter multiple divisions, giving rise to calli or to embryo-like structures and even to diploid plants.

Another way established in anther response to *in vitro* culture appeared to be direct induction of embryogenic structures with a fairly rapid ongoing development embryo. Microspores dedifferentiation and formation of embryonic structures is supplemented by increased cell proliferation.

Our cytological investigation confirmed the origin of these calli and showed their microspore origin.

For evaluation of the explants potential the application of histological studies of anthers with different response reaction had been proposed (Figure 3).

Structures derived from microspores with embryogenic potential after several reproductive cycles, pass to the globular stage, constituted by the meristematic cells (Figure 3A, 3B). Meristematic centres are characterized by cells compact arrangement, hyperchromatic cytoplasm due to high content of organelles and intracellular compounds, major centrally located nuclei, lack of central vacuole. Histological studies showed that globular structures originated at sub-marginal region and were covered by the epidermis. The somatic embryos formed independently or very close and appeared to be linked by their basal region.

In late developmental stage, the globular structures that formed embryos, exhibited polarity (apical and basal regions) (Figure 3E, 3F), precocious tracheary differentiation appeared, of which elements were found among the meristematic cells (Figure 3G, 3H).

Further sub-cultivation conducted to embryo differentiation or loss of cell adhesion and cell vacuolization (Figure 3D).

We observed that the morphogenetic structures were related to accumulation of storage of substances, mainly starch (Figure 3I, 3J) and phenolic compounds (Figure 3J).

It is known that an important prerequisite for the potency expression is the nutrient substrate, such as starch, which serves as a source of energy for metabolic needs at the initial stage. The accumulation of phenolic compounds can be attributed to the protection of IAA *in vitro* from IAA-oxidase. Consequently, the genotype with maximum *in vitro* protection for IAA is regarded as the best genotype for androgenesis (Delalade et al., 1996).

According to the obtained results, the anthers response to *in vitro* conditions was expressed through embryogenic or callus induction or lack of response, followed later by necrosing processes.

ANOVA revealed significant variation of the type of reaction (callus or embryogenesis) (at confidence level 99.9%) (Table 2). Anther response type had maximum power of influence that value was 77.7%. Genotype, with a power of influence about 9.7%, was also a significant source of variation (at 95% confidence level).

Regenerants obtained from embryogenic structures showed *albino* mutation in proportion of 95.8%. Normal, green forms were obtained only for the cv. Unirea. Low success in obtaining of green plants for analysed barley varieties was influenced

primarily by genotypic peculiarities. Limited capacity of haploid generation was determined by a complex of interrelated genetic and environmental factors.

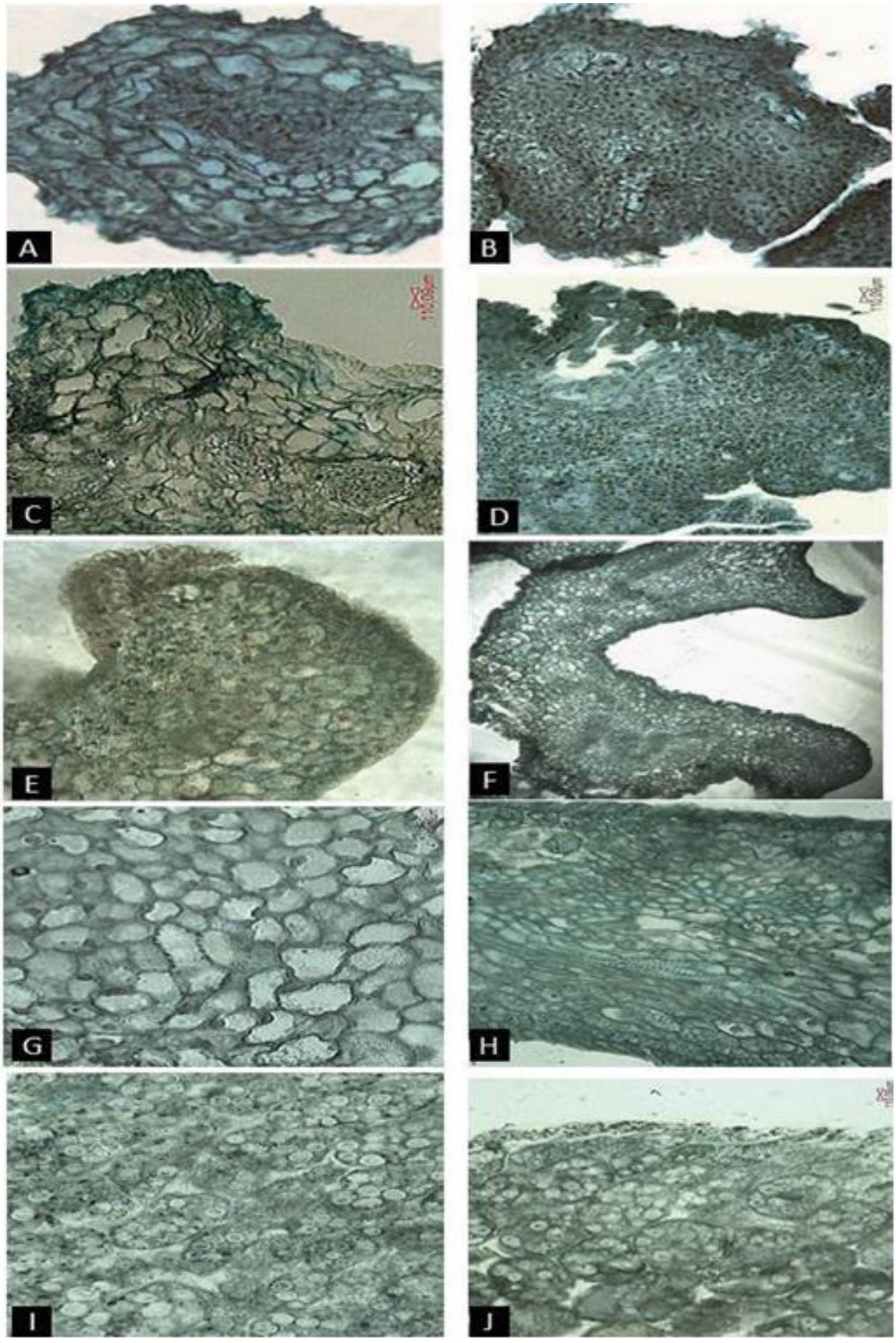

Figure 3. Histological aspect of structures derived from anthers culture: cells structure at globular stages (A-B), differentiation of embryo-like structures (C), somatic embryos formation, exhibiting polarity (D, E-F), tracheary differentiation at pre-embryonary stage (G-H), morphogenetic structures with accumulation of starch and phenolic compounds (I-J)

Table 2. Analysis of variance of anthers reaction to culture medium within spikes of different barley genotypes

Source of variance	Contribution of the source of variance (%)	Degrees of freedom	Sum of squares	Mean square	F-Ratio	P-value
A: Genotype	9.70	3	2045.54	681.846	3.60*	0.0143
B: Culture medium	0.93	2	19.5645	9.78226	0.52	0.5975
C: Reaction types	77.75	2	16389.8	8194.92	432.49***	0.0000
D: Spike	1.51	14	317.248	24.4037	1.29	0.2113
Total	21079.5	242				

The estimated value of correlation between studied factors (genotype, culture medium response type, and spike) showed no interrelationship, except only for the pair of characters "culture medium - type response", indicating the complexity of androgenetic processes.

As results of optimised technique for initiation *in vitro* embryogenesis, we established that cold stress (+4°C) in complex with mannitol starvation generate reorganization involving anther wall cells (tapetum, endothecium) and microspores reprogramming.

CONCLUSIONS

Cytological evaluation of anther derived-structure revealed the formation of calli and embryogenic structures, microspore embryogenesis providing a complex morphogenetic pathway, not only dependent on the stage factors, influenced dedifferentiation.

On base of histological evaluation we described a set of restructuring, which conducted to embryo differentiation from globular stage; pre-embryonic structures to embryo formation. These reorganizations may be inhibited and stopped at any development stage. The androgenic capacity was found related to accumulation of storage of substances and phenolic compounds.

Evaluation of morphological and structural features of morphogenetic structures initiated from barley anthers, proved the complexity of androgenetic process, dependent not only on the stage of generating microspore dedifferentiation, but including pretreatment conditions and the genotype peculiarities.

REFERENCES

Bal, U., Shariatpanahi, M.E., Castro, A.J., Emery, D., Clément, Ch., Dehestani-Ardakani, M., Mozaffari, K. and Touraev, A., 2012. *Pseudo-embryogenic Structures in Anther and Isolated Microspore Cultures in vitro: a Cautionary Guide*. Czech J. Genet. Plant Breed, 48, (2): 51-60.

Cistue, L., Valles, M.P., Echavarri, B., Sanz, J.M. and Castillo, A., 2003. *Barley anther culture*. In: Maluszynski, M., Kasha, J., Forster, B.P. and Szarejko, I. (eds.), Doubled haploid production in crop plants. Ed., Kluwer Academic publishers, Dordrecht, Boston, London: 29-34.

Clewer, A.G., Scarisbrick, D.H., 2001. *Practical statistics and experimental design for plant and crop science*. John Wiley & Sons, Ltd., Chichester, UK.

Chen, L.J., Zhu, X.Z., Gu, L. and Wu, J., 2005. *Efficient callus induction and plant regeneration from anther of Chinese narcissus (Narcissus tazetta L. var. chinensis Roem)*. Plant Cell Reports, 24: 401-407. DOI: 10.1007/s00299-005-0958-4.

Chu, C.C., 1978. *The N6 medium and its application to anther culture of cereal crops*. In: Proc. Symp. of Plant Tissue Culture, Science Press, Beijng, China: 43-50.

D'yachuk, T.I., Khomyakova, O.V. and Dugina, T.O., 2010. *Cytological evidence of sporophyte development of microspores in culture of triticale anthers without cold treatment*. Sel'skokhozyaistvennaya Biologiya [Agricultural Biology], 5: 61-65.

Delalonde, M., Barret, Y. and Coumans, M.P., 1996. *Development of phenolic compounds in maize anthers (Zea mays) during cold pretreatment prior to androgenesis*. Journal of Plant Physiology, 149: 612-616. DOI:10.1016/S0176-1617(96)80342-7

Gribaudo, I., Gambino, G. and Vallania, R., 2004. *Somatic embryogenesis from grapevine anthers: the optimal developmental stage for collecting explants*. American Journal of Enology & Viticulture, 55: 427-430.

Hoekstra, S., van Bergen, S., van Brouwershaven, I.R., Schilperoort, R.A. and Wang M., 1997. *Androgenesis in Hordeum vulgare L.: Effects of mannitol, calcium and abscisic acid on anther pretreatment*. Plant Sci., 126: 211-218. DOI:10.1016/S0168-9452(97)00096-4.

Hoekstra, S., van Zijderveld, M.H., Louwerse, J.D., Heidekamp, F. and van der Mark, F., 1992. *Anther and microspore culture of Hordeum vulgare L. cv. Igri*. Plant Sci., 86: 89-96. DOI:10.1016/0168-9452(92)90182-L.

Huang, B. and Sunderland, N., 1982. *Temperature-stress pretreatment in barley anther culture*. Ann. Bot., 49: 77-88.

Hunter, C.P., 1988. *Plant regeneration from microspores of barley, Hordeum vulgare L*. Ph. D. Thesis, Wye College, Univ. of London.

Jacquard, C., Wojnarowiez , G. and Clement, C., 2003. *Anther culture in barley*, In: Maluszynski, M., Kasha, J., Forster, B.P. and Szarejko, I. (eds.), Doubled haploid production in crop plants. Ed. Kluwer Academic Publishers, Dordrecht, Boston, London: 21-27.

Islam, S. and Tuteja, N., 2012. *Enhancement of androgenesis by abiotic stress and other pretreatments in major crop species*. Plant Sci., 182: 134-144. DOI: 10.1016/j.plantsci.

Kruczkowska, H., Pawowska, H. and Skucinska, B., 2002. *Influence of anther pretreatment on the efficiency of androgenesis in barley*. J. Appl. Genet., 43(3): 287-296.

Maraschin, S. F., W. de Priester, Spaink, H. P. and Wang, M., 2005. *Androgenic switch: an example of plant embryogenesis from the male gametophyte perspective*. J. Exp. Bot., 56: 1711-1726. DOI:10.1093/jxb/eri190.

Powell, W., 1998. *Diallel analysis of barley anther culture response*. Genome, 30: 152-157.

Robert-Oehschlager, S.L. and Dunwell, J.M., 1990. *Barley anther culture: pretreatment on mannitol stimulates production of microspore derived embryos*. Plant Cell, Tissue and Organ Culture, 20: 235-240. DOI :10.1007/BF00041887.

Salas, P., Rivas-Sendra, A., Prohens, J. and Seguı´-Simarro, J.M., 2012. *Influence of the stage for anther excision and heterostyly in embryogenesis induction from eggplant anther cultures*. Euphytica, 184: 235-250. DOI:10.1007/s10681-011-0569-9.

Shariatpanahi, M.E., Bal, U., Heberle-Bors, E., Touraev, A., 2006. *Stresses applied for the re-programming of plant microspores towards in vitro embryogenesis*. Physiol. Plant, 127: 519-534. DOI:10.1111/j.1399-3054.2006.00675.x.

Soriano, M., Li, Hui and Boutilier, K., 2013. *Microspore embryogenesis: establishment of embryo identity and pattern in culture*. Plant Reprod., 26: 181-196. DOI:10.1007/s00497-013-0226-7.