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USE OF MANNITOL FOR COLD PRETREATMENT OF ISOLATED SPIKES AND AS A COMPONENT OF NUTRIENT MEDIUM FOR SPRING BARLEY ANTHER CULTURE *IN VITRO*

Aim. The investigation was aimed to compare the efficiency of mannitol as a component of spike cold pretreatment solution and of medium for spring barley haploid production via anther culture in vitro. ***Methods.*** Field-grown plants of two genotypes with a contrast androgenic capacity were used as donors. Cut tillers were pretreated in water at 4^0 C for 5 days, while aseptically isolated spikes were kept in 0.3 M mannitol for 10 days at the same temperature. Isolated anthers were inoculated on inductive media containing N6 macro-, MS micronutrients, organic supplements, maltose (9.0%); the media with and without 0.1M mannitol differed in solidifying agents. ***Results***. In highly-responsive line DH00-126, there was an almost two-fold increase in mmgreen plant regeneration frequency (from 48 to 58 plants per 100 anthers) in all experimental variants in comparison to the control. In recalcitrant variety Mebere, the combination of tiller pretreatment in water with anther cultivation on inductive starch-gelling media supplemented with 0.1 M mannitol resulted in the highest green plant yield. ***Conclusions.*** Both methodical approaches were proved to be applicable to increase the efficiency of haploid production in spring barley anther culture *in vitro*.

Keywords: *Hordeum vulgare* L., anther culture *in vitro*, cold pretreatment, mannitol, agar, chemically modified starch.

Microspores are known to be highly specialized cells and precursors of the male gametophyte in high plants. They derive from tetrads which possess haploid chromosome number. Tetrads in their turn are formed after meiosis from pollen mother cells. During gametogenesis, some morphological changes such as formations of pore, large vacuole and exine take place in microspore. These changes determine a subsequent microspore fate to develop through two successive mitotic divisions into pollen grain containing vegetative nucleus and gametes [1].

In anther or microspore culture *in vitro* microspores continue to play a role of precursors. However, in this case, their development result in haploid and doubled haploid plant production. In particular, instead of two divisions multiple ones occur. These events lead to multicellular structure formation inside microspores. After liberation from exine, these multicellular structures develop into calluses or embryoids capable to plantlet regeneration [2].

It is evident that described pathway of morphogenesis *in vitro* is abnormal and based on reprogramming of microspore development. Hence, it needs artificial induction by certain external stress factors. On the basis of studying the efficiency of different methodical approaches potentially suitable for enhancement of embryo-like structure induction and plant regeneration in anther culture *in vitro*, many methods of tiller, inflorescence and anther pretreatments were developed. In cereals, including barley and wheat, pretreatment of tillers or spikes at low positive temperatures and different cold periods [3] as well as immersion of spikes or anthers in mannitol solution were frequently applied [4, 5]. In addition, mannitol was successfully used in inductive media for barley haploid production [6]. Nevertheless evaluation of cold and mannitol pretreatment effects on androgenesis *in vitro* showed controversial results due to diverse reasons (genotype, conditions of donor plant growing, medium composition), and anthers isolated from freshly cut inflorescence sometimes exceed pretreated ones in morphogenic reaction [7], these experimental stress treatments remain the important elements of haploid technologies [8]. Furthermore, investigations aimed to ascertain mechanisms of different treatments on cell structure and on genome activity were carried out using modern methods of cytology, biochemistry and molecular genetics. Early

reports showed that chilling decreased microspore stage progression towards pollen grains and enhanced the number of symmetrical nucleus division favorable for a direct embryogenesis [9]. Recent studies indicated that treatments at low positive temperatures led to structural changes in cytoskeleton [10], induced programmed cell death and autophagy [11] as well as affected microspore DNA demethylation, which was considered to be a prerequisite for switching microspore development program [12].

The purpose of this investigation was to compare the efficiencies of mannitol usage in isolated spike cold pretreatment solution and as a supplementary component of the inductive medium for spring barley haploid production via anther culture *in vitro.*

Materials and methods

Two spring barley genotypes with a contrast capacity to androgenesis *in vitro* were used as plant material. Highly-androgenic line DH00-126 is characterized both by high frequencies of embryoid and callus induction as well as green plant regeneration in anther culture *in vitro.* Mebere is a recalcitrant variety with a low yield of green plants but with a high rate of albino plant regeneration.

Plants were grown on experimental plot. Climatic conditions were favorable for obtaining vigorous plants at reproductive stage. Tillers were cut when microspores had reached middle or late vacuolated phases of their development. Microspore developmental stage was estimated on squash preparations of anthers stained with 2% carmine solution in 45% acetic acid.

Freshly cut tillers were divided into two groups. The first one was directly subjected to cold pretreatment. In particular, tillers were kept rinsed in tap water at 4ºC in the dark in a refrigerator for 5 days. The second group of the tillers was sterilized with 70% ethanol for 10−15 min and used for spike removal in aseptic conditions. Then spikes were kept in Petri dishes (100 mm diameter, sealed with Parafilm) in 0.3M mannitol solution at 4ºC for 10 days. Optimal duration of the pretreatments were determined in our previous investigations [13]. Tillers pretreated in water were sterilized before spike removal and anther excision as described above.

Improved NMSmod2 inductive medium used as a basic one contained N6 macro- [14], MS micronutrients [15], 100 mg l^{-1} myo-inositol, 1 mg l^{-1}, thiamine-HCl, 0.5 mg l^{-1} pyridoxine-HCl, 0.5 mg l^{-1} nicotinic acid, 200 mg l^{-1} glutamine, 100 mg l^{-1} proline, 100 mg l^{-1} alanine, maltose (9.0 %), pH 5.8. The control and experimental variants of medium were differed in solidifying agents (agar – 0.8 % or chemically modified starch D5a-M – 11.0 %). Starch-solidified media were also different in 0.1 M mannitol addition. The starch preparation was produced and kindly supplied by P.G. Dulniev.

Experimental design contained control and four experimental variants. Anthers excited from material pretreated in water were inoculated on agar-solidified inductive medium (control), on the starch solidified medium (variant 1) as well as on the starch-solidified medium supplemented with 0.1 M mannitol (variant 2). Anthers excised from the spikes pretreated in 0.3 M mannitol were cultivated on the media solidified with agar (variant 3) and with chemically modified starch D5a-M (variant 4). Tubes contained anthers were incubated at 24−25ºC in the dark. Observations were carried out starting from the 20th day of cultivation.

Obtained embryoids, globular structures and calluses were transferred to regenerative medium contained MS macro- and micronutrients, 100 mg l^{-1} myo-inositol, 200 mg l^{-1} glutamine, 0.5 mg l^{-1} thiamine-HCl, 0.5 mg l^{-1} pyridoxine-HCl, 0.5 mg l^{-1} nicotinic acid, 0.5 mg l^{-1} BAP, 0.05 mg l^{-1} NAA, 3.0 % sucrose, 0.8 % agar, pH 5.8. The efficiencies of androgenesis *in vitro* was determined as the percentage of morphogenic anthers, i.e. anthers with calluses or embryoids appeared on their surfaces, and as a number of green plants per 100 inoculated anthers.

The significance of differences between experimental variants and the control ware evaluated on the base of LSD_{05} obtained from ANOVA for qualitative traits using «Microsoft Office Excel 2010» program.

Results and discussion

Results of observations showed a strong genotypic dependences both in morphogenic structure induction and in plant regeneration frequency. Effects of pretreatment mode and of inductive medium as well as combination of these factors were also obvious, especially for green plant regeneration (Table).

Table. Androgenesis *in vitro* in spring barley anther culture in dependence to genotype, mode of cold pretreatment and gelling agents of nutrient media for anther cultivation

Experimental variant (Mode of pretreatment at 4 °C, gelling agent of medium, mannitiol addition)	Number of inoculated anthers	Morphogenic anthers		Green plant regeneration	
		Number	%	Number	Number per 100 anthers
DH 00-126					
Tillers, water, 5 days, agar (control)	410	216	52,68	110	26,82
Tillers, water, 5 days, D5a-M	438	194	44,29	226	51,60
Tillers, water, 5 days, D5a-M+0.1 M mannitol	394	208	54,79	214	54,31
Spikes, 10 days, 0.3 M, agar	404	222	54,95	238	58,91
Spikes, 10 days, 0.3 M, D5a-M	406	206	50,73	195	48,03
LSD_{05}	–	–	6,83	–	6,67
Mebere					
Tillers, water, 5 days, agar (control)	668	144	21,56	7	1,05
Tillers, water, 5 days, D5a-M	509	166	32,61	14	2,75
Tillers, water, 5 days, D5a-M+0.1 M mannitol	506	101	19,96	35	6,91
Spikes, 10 days, 0.3 M, agar	639	160	25,03	17	2,66
Spikes, 10 days, 0.3 M, D5a-M	497	56	11,26	2	0,40
LSD_{05}	–	–	3,03	–	1,73

In a highly-responsive line DH00-126, the number of morphogenic anthers varied from 44.3 to 54.9 %. The lowest percentage was obtained when D5a-M starch-solidified medium was used for inoculation of anthers isolated from tillers kept in tap water for 5 days. In other experimental variants and in the control, differences in the number of morphogenic anthers were not significant and exceeded 50 %. Notably, that in all experimental variants almost two-fold increases in green plant regeneration efficiency were reached in comparison to the control. It was shown positive effect of chemically modified starch on plant regeneration after tiller pretreatment in water. At the same time, when 0.3 M mannitol was used for spike pretreatment higher frequency of green plant regeneration was obtained on agar solidified medium (58.9 instead of 48.1 plants per 100 cultivated anthers). As to addition of mannitol to the inductive medium solidified with chemically modified starch, this improvement promoted androgenic structure induction but didn't significantly affect green plant regeneration.

In a recalcitrant variety Mebere, morphogenic anther frequencies ranged from 11.3 to 32.6 % that was significantly lower than in DH00-126 regardless of treatments and media used. The highest frequency in morphogenic structure induction was obtained when anthers excised from tillers pretreated in water were cultivated on the starch-solidified medium. At the same time, combination of spike pretreatment in 0.3 M mannitol solution and subsequent anther inoculation on the starch-gelling medium resulted in drastically decrease in the number of morphogenic anthers in comparison to the control and to other variants of the experiment. The highest yield of green plants (6.9 per 100 anthers) was obtained when tillers were pretreated in water, and medium containing chemically modified starch and 0.1 M mannitol was applied.

It was found that most of regenerated plants were obtained by transferring of calluses, proem-

bryo globular structures and embryoids to regenerative medium. However, differentiated embryoids were also capable to germinate on the inductive media. Notably, that this process was more efficient on agar solidified media regardless of the mode of cold pretreatment (Fig. 1–4). On the starch-solidified inductive medium, embryoid germination occurred only after spike pretreatment in 0.3M mannitol solution. Nevertheless, in the latter experimental variant plant regeneration rate was lower than in other ones, regenerated plants were characterized by normal morphology without any traits of hyperhydrocity. On agar-solidified media active growth of callus with a low regenerative potential occurred. Furthermore, this process was not suppressed by spike pretreatment in 0.3 M solution. In order to prevent a decrease in callus and proembryo abilities to plant regeneration, they were transferred to regenerative medium a soon as possible.

Generally, obtained data confirmed the importance of temperature and osmotic stresses for induction of morphogenesis in spring barley anther culture *in vitro*. Cold pretreatment of spikes in water for 5 days (short-term storage) as well as in 0.3 M mannitol solution for 10 days (middle-term storage) allowed increase the duration of anther inoculation period on the inductive media that is very important for haploid production from hybrid breeding material.

The results of our investigations also revealed the best combinations of pretreatment mode and gelling agent of medium. Particularly, agar-solidified media may be recommended after pretreatment of plant material in 0.3 M mannitol solution.

When spikes before anther isolation were subjected to cold pretreatment in water, medium solidified with chemically modified starch was appeared to be more suitable for anther cultivation. In a recalcitrant genotype, positive effect of 0.1 M mannitol addition to the medium on green plant regeneration was found.

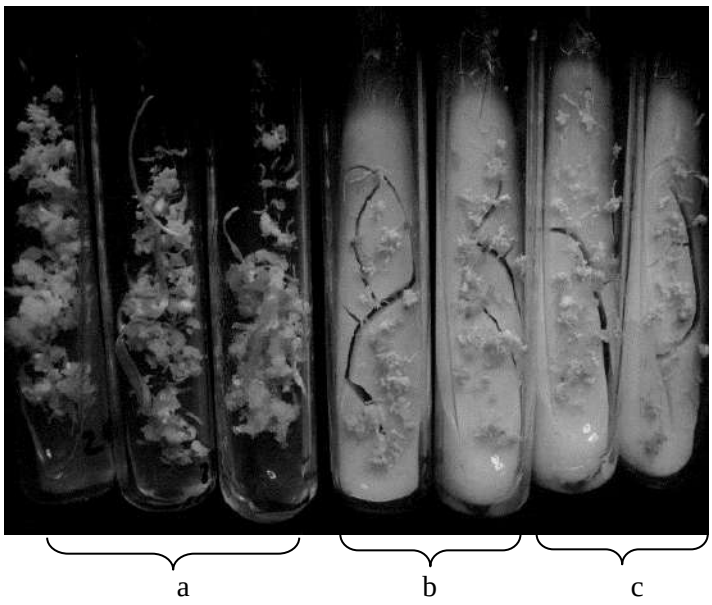

Fig. 1. Induction of morphogenic structures and plant regeneration in anther culture *in vitro* in spring barley line DH00-126 on the inductive media differed in gelling agents after cold pretreatment of donor plant tillers in water at 4°C for 5 days. a) 0,8% agar (control); b)11,0% D5a-M; c) 11,0 % D5a-M+0,1 M mannitol.

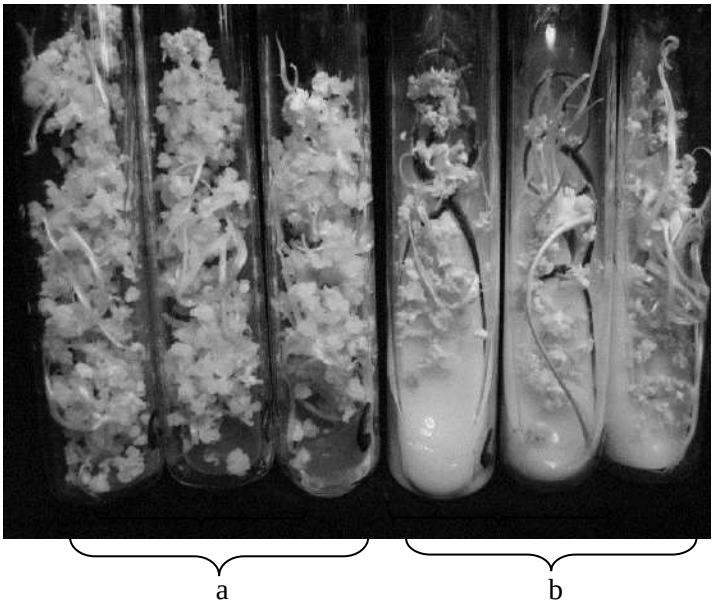

Fig. 2. Induction of morphogenic structures and plant regeneration in anther culture *in vitro* in spring barley line DH00-126 on the media differed in gelling agents after cold pretreatment of donor plant spikes in 0.3 M mannitol solution at 4°C for 10 days. a) 0,8% agar; b)11,0% D5a-M.

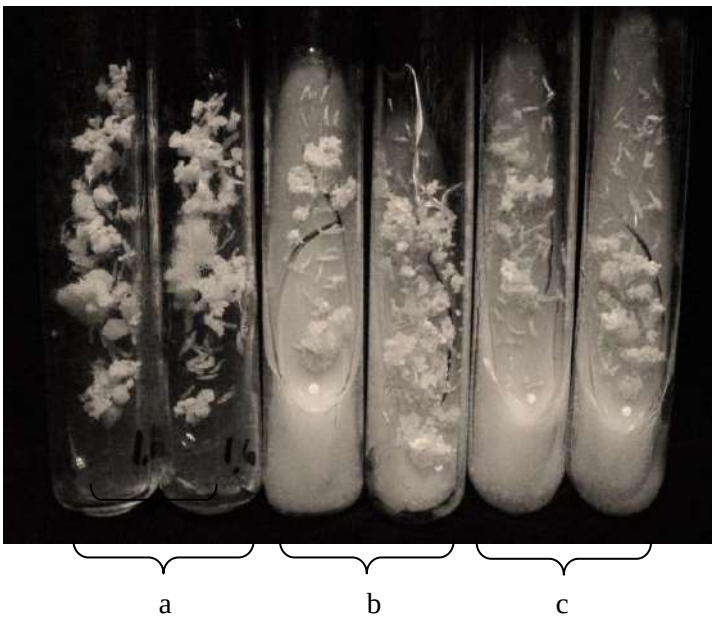

Fig. 3. Induction of morphogenic structures and plant regeneration in anther culture *in vitro* in spring barley variety Mebere on the inductive media differed in gelling agents after cold pretreatment of donor plant tillers in water at 4°C for 5 days. a) 0,8% agar (control); b)11,0% D5a-M; c) 11,0 % D5a-M+0,1 M mannitol.

Conclusions

For the first time, a comparative study on the efficiency of different ways of mannitol usage for improvement of spring barley haploid production through anther culture *in vitro* was carried out. It was shown genotypic dependences in response to the mode of cold pretreatment and gelling agent of media. Regardless of the applied technological elements and their combinations, androgenic capacity ranks of genotypes remained unchanged. Both methodical approaches – mannitol use for spike pretreatment and as a component of medium – were proved to be applicable for increase the efficiency of haploid production in spring barley anther culture *in vitro*.

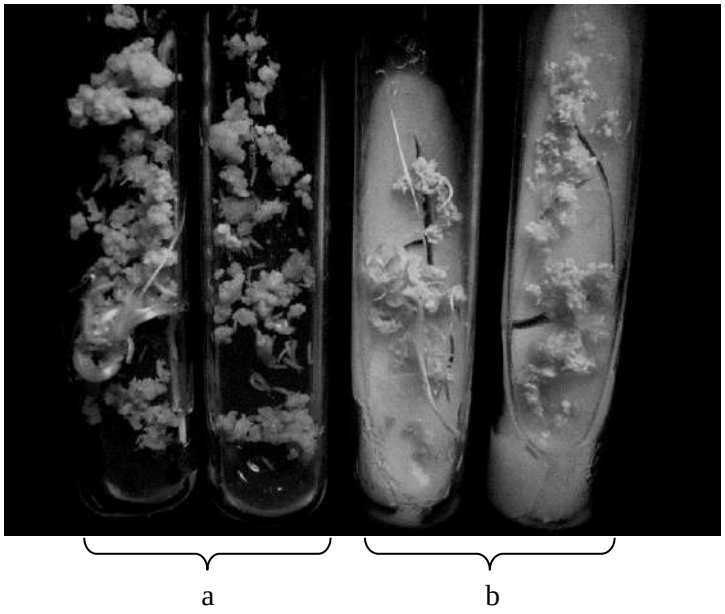

Fig. 4. Induction of morphogenic structures and plant regeneration in anther culture *in vitro* in spring barley variety Mebere on the media differed in gelling agents after cold pretreatment of donor plant spikes in 0.3 M mannitol solution at 4°C for 10 days. a) 0,8% agar; b)11,0% D5a-M.

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ВИКОРИСТАННЯ МАНІТОЛУ ДЛЯ ПОПЕРЕДНЬОЇ ХОЛОДОВОЇ ОБРОБКИ ІЗОЛЬОВАНОГО КОЛОССЯ ТА ЯК КОМПОНЕНТА ЖИВИЛЬНОГО СЕРЕДОВИЩА ДЛЯ КУЛЬТУРИ ПИЛЯКІВ *IN VITRO* ЯРОГО ЯМЕНЮ

Мета. Порівняння ефективності додавання манітолу до розчину для попередньої холодової обробки ізольованого колосся та до живильного середовища для одержання гаплоїдів ярого ячменю у культурі пиляків *in vitro*. ***Методи***. Донорні рослини вирощували у польових умовах. Пагони було вміщено у воду і витримано впродовж 5 діб за температури +4 °С. Ізольоване колосся було попередньо оброблене у 0,3 М розчині манітолу впродовж 10 діб за тієї ж температури. Пиляки було інокульовано на індукційні середовища, які містили макро- та мікроелементи відповідно за прописами N6 та MS, органічні компоненти, мальтозу ((9,0 %), і різнилися загусниками та додаванням 0,1 М манітолу. ***Результати.*** У чутливої до андрогенезу *in vitro* лінії ДГ00-126 досягнуто майже дворазове зростання кількості зелених рослин-регенерантів в усіх дослідних варіантах (до 58 рослин на 100 пиляків) порівняно з контролем. У сорту Mebere з низькою андрогенною здатністю найвищий вихід зелених рослин одержано за поєднання попередньої обробки пагонів у воді з подальшим культивуванням пиляків на середовищі з 0,1 М манітолом і хімічно модифікованим крохмалем. ***Висновки***. Обидва методичні підходи є придатними для підвищення ефективності одержання гаплоїдів ярого ячменю у культурі пиляків *in vitro*.

Ключові слова: *Hordeum vulgare* L., культура пиляків *in vitro*, попередня обробка, манітол, агар, хімічно модифікований крохмаль.