STUDY OF FACTORS AFFECTING SPOROPHYTIC DEVELOPMENT OF ISOLATED DURUM WHEAT MICROSPORES

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Abstract

A microspore culture technique is used to produce homozygous lines of agronomic interest in a single generation. Several factors affecting the efficiency of *in vitro* sporophytic development of durum wheat microspores such as genotype, stress preatreatment, density and co-cultivation of isolated microspore and induction medium were studied in order to develop a protocol for dihaploid plants production. Strong genotype dependence in *in vitro* response and callus/embryoid capacity was observed. Cultivated microspores developed into macrostructures only after cold pretreatment of the collected spikes at +4°C for 4 weeks. The best results were achieved at density 10000 microspores/ml on the mod. Chu Medium (Chu et al., 1990) + 0.5 mg/l BAP + 0.5 mg/l 2,4D + 90 g/l maltosa with ovaries co-cultivation. A lot of macrostructures developed to embryoids, but only a few produced poorly-developed shoots – most of them were albinos.

Key words: durum wheat, dihaploids, microspore culture

INTRODUCTION

The production of haploid/doubled haploid (DHs) plants from gametic cells i.e. microspores is advantageous in plant breeding, genetic manipulation and in many areas of basic research in plant development biology (Zheng, 2003).

DH production through isolated microspore culture advanced rapidly in recent years in parallel with the brooding of understanding of the genetic, cellular, biochemical and molecular events associated with microspore embryogenesis (Seguí-Simarro and Nuez, 2008). After the optimization of the major factors affecting the efficiency of *in vitro* sporophytic development of microspores such as stress preatreatment, methods of isolation and optimal culture conditions it is now possible to produce thousands of green plants from microspores isolated from a single wheat spike (Touraev *et al.*, 1996; Hu and Kasha, 1997; Liu *et al.*, 2002). Tetraploid durum wheat (AABB) exhibits much lower androgenetic responsiveness than hexaploid common wheat does (Mentewab and Sarrafi, 1997). Until recently it is consider that durum wheat is recalcitrant species, because of it low response to culture manipulation with a few embryogenic microspores induced, high rate of embryogenic development abortion and high albino frequencies (Otani and Shimada, 1994). In the last years significantly improvement in embryogenesis

induction and green plants regeneration in microspore culture has been reported (Labbani et al., 2007, Cistué et al., 2009; Ayed et al., 2010).

The main objective of this investigation is to study several factors affecting the efficiency of *in vitro* sporophytic development of durum wheat microspores such as genotype, stress preatreatment, density and co-cultivation of isolated microspore and induction medium in order to develop a protocol for dihaploid plants production.

MATERIALS AND METHODS

The experiments were conducted in Biocenter Klein Flottbek – University of Hamburg. Two Bulgarian durum wheat cultivars – Saturn and Progres and two breeding lines were used in the investigation. The donor plants after the vernalisation at 4° C for 4 weeks were grown under controlled conditions in a green house at 16-18/14-16° C day\night, light – 10 000 – 16 000 lux and relative humidity 70-90 %. The spikes were harvested when the anthers of the middle part of the spikes contained the microspores from mid- to late-uni-nucleate stage. The spikes were subjected to cold pretreatment from one to four weeks before using them for isolated microspores culture. A control without pretreatment was used, too (table 1). The spikes were sterilized with calcium hypochlorite 2% for 10 min before pretreatment and were stored in two –compartment Petri dishes (Greiner, Frickenhausen, Germany) with destiliert water in one compartment and the spikes – in other one. Microspores were isolated via microblending (Microblender - Eberbach Corporation, Ann Arbor, Michigan, USA) of segmented spikes according to the method described by Jaehne-Gaertner and Loerz, 1999. The total numbers of microspores were determined with a haemocytometer(Fuchs-Rosenthal) and theirs viability – with fluorescein diacetate under UV-Mikroskop. Two different basic media: K 99 (Kumlehn, nicht veröfentlicht) und Chu et al., (1990) with addition of 0,25 M maltose instead glucose were used as induction medium (table 1). The microspores were incubated in nine different density – numbers of microspores on 1ml medium with and without co-cultivation of ovaries of studied durum wheat genotypes and microspores of barley model cultivar Igri (table 1) adjusted by adding induction medium in 35×10 mm Petri dishes in darkness at 26° C. After 4 to 6 weeks after beginning of cultivation the embryo-like-structure and calluses were transferred to regeneration medium (medium B) with reduced maltose content (45 g/l maltose) solidified with 0.6 % gelreit and further cultivated at 24° C under 16 h photoperiod.

RESULTS AND DISCUSSIONS

The first *in vitro* division of durum wheat microspores started still after 24 hours since the beginning of cultivation. Symmetric and asymmetric first division were observed. The big part of microspores plasmolysed without to divide or degenerated after first *in vitro* divisions irrespective of the existence of many big and able to divide microspores or "star-like" structures. Three days after the

beginning of cultivation 10 to 40 % of all microspores continued to divide further, but 3 days later this percent decreased to 10 - 0.5 % depending on the genotype. A limited proportion between responded and dead microspores led later to completely plasmolysis and degeneration of whole culture at two of utilized genotypes.

In table 2 are presented only the positive results from the cultivation of durum wheat microspores. Microscopic and macroscopic structures (embryoids/ calluses) were induced only at cultivar Saturn and breeding line M 6129. Cultivar Saturn responded better with 0, 04 to 0.34 % of callus/embryoids induction depending on the density of microspores and induction medium (figure 1). Another two genotypes were not responsive. The importance of genotype on the androgenetic ability is well documented and is confirmed in our study.

On media A was not observed sporophytic development of durum wheat microspores. Although this induction medium was very suitable for barley microspores and many green plants were regenerated at barley cultivar Igri that serves as a control in our study. On media B were induced more calluses/ embryoids in comparison with media C. Both variants of medium differ only in terms of maltose concentration and therefore the higher concentration supports better the sporophytic development of durum wheat microspores.

The microspores developed to macroscopic structure only after one prolonged cold pretreatment of the collected spikes at +4°C for 4 weeks. Our results are in agreement with other studies in durum wheat (Labbani et al., 2007; Ayed et al., 2010) and emphasized that pre-treatment is one of the most important factor switching normal gametophytic pathway of microspores to sporophytic one.

It was not possible further sporophytic development of durum wheat microspores to be achieved without one co-cultivation of isolated microspore with ovaries or barley microspores. The best results were received on media B with co-cultivation of 5 immature ovaries per ml media. The crucial role of ovary co-culture for success of wheat microspore culture was found in other study, too (Hu and Kasha, 1997;Cistué et al., 2009), whereas at barley the co-cultivation is not so important factor (Lu et al, 2008).

Microscopic structures were observed at density of microspores from 5000 to 50000 per ml media. Most macroscopic visible structures - 0.34 % were achieved at density 10000 microspores per ml media in cultivar Saturn. Satisfactory macroscopic structures were obtained at density 5000 and 2000 microspores per ml, too. These results illustrate that culture density is another essential factor for success of microspore culture, and has influence on both proportion of responsive microspores and normal development of embryoids.

Although the embryoids were induced, most of them failed to regenerate and only a few - produced poorly-developed albino shoots. This fact is not unexpected because hitherto albinism was the major problem in durum wheat anther and microspore culture.

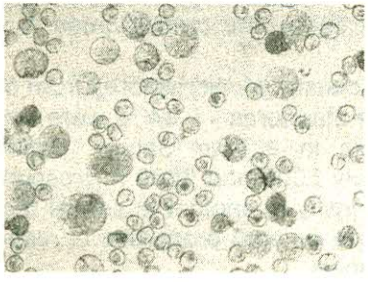
The present study is a promising start for the production of doubled haploid durum wheat plants in Bulgaria via isolated microspore culture. The main factors affecting in vitro development of microspores were investigated. However, further

studies on the optimization of both responded microspores and regeneration ability of induced embryoids are needed.

Table 1.

Investigated factors in microspore culture of durum wheat

Factors studied	Variants
Genotype	Saturn, Progres, M-6129, M–5918
Duration of cold pretreatment of spikes at +4°C (days)	0, 7, 14, 28
Density of microspores in 1 ml media	100, 500, 1000, 5000, 10000, 20000, 25000 50000, 100000
Induction medias	**A** - K98 (Kumlehn, not published) + 1 mg/ml BAP + 90 g/l maltose pH 5.8, 0.467 osmol/kg **B** - mod. Chu Medium (Chu et al., 1990) + 0.5 mg/l BAP + 0.5 mg/l 2,4D + 90 g/l maltose, pH 5.4, 0,345 osmol/kg **C** – mod. Chu Medium (Chu et al., 1990) + 0.5 mg/l BAP + 0.5 mg/l 2,4D + 20g/l maltose, pH 5,4
Co-cultivation of induction medium	ovaries - 0 , 1, 5, 10 barley microspores



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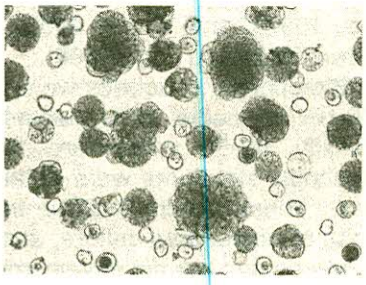

b

Figure 1. In vitro development of durum wheat microspores cv. Saturna – a - dividing microspores after 7 days in culture b - microscopic structure after 14 days in culture

Table 2.

Kallus/embryoids induction in durum wheat microspore culture (only positive results)

Genotyp	Inductions -medium	Duration of cold pretreat-ment	Density of microspores per ml	Ovaries per ml	Callus/embryoids induction	
					Per petri dish	%
Saturn	B	28	5000	5	13	0.26
	B	28	10000	5	34.3	0.34
	B	28	20000	5	55	0.28
	B	28	25000	5	30.5	0.12
	B	28	50000	5	40	0.08
	C	28	10000	5	15	0.15
	C	28	5000	5	12	0.24
	C	28	25000	5	10.5	0.04
M-6129	B	28	20000	5	23.5	0.12
	B	28	50000	5	7	0.02
	C	28	25000	5	8	0.03
	C	28	50000	5	9	0.02

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Acknowledgment to the Deutscher Akademischer Austausch Dienst (DAAD Referat 324, Kennziffer A/03/10404) for financial support.