GENOTYPIC COMPETENCY IN EMBRYOGENESIS AT *BRASSICA OLERACEA* MICROSPORES CULTIVATED *IN VITRO*

STUDII PRIVIND COMPETENŢA GENOTIPICĂ ÎN EMBRIOGENEZA MICROSPORILOR DE *BRASSICA OLERACEA* CULTIVAŢI *IN VITRO*

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Abstract. *In Brassica species the obtaining of doubled haploid plants is a key tool for the production of commercial F1 hybrids. Among the many methods employed for this purpose, the culture of isolated microspores offers the opportunity to generate double haploid embryos starting from single haploid cells, thus assuring the genetic purity of haploid plants obtained. The effect of donor genotype on the success of isolated microspores cultures was investigated by many authors in different cultivated species, the results demonstrate that it plays a key role. Genetic factors not only influence the viability of microspores and frequency of normal embryos but also the rate of plant regeneration. In this paper, a complete screening of microspores viability as well as embryo development under the influence of genotype is presented.*
Key words: haploids, cabbage, *in vitro*, regeneration, plants

Rezumat. *La speciile de Brassica, obţinerea de plante dublu haploide este un instrument cheie pentru producerea de hibrizi comerciali F1. Dintre numeroasele metode folosite în acest scop, cultura de microspori izolaţi oferă posibilitatea de a genera embrioni dublu haploidici pornind de la celule haploide unice, asigurând astfel puritatea genetică a plantelor haploide obţinute. Efectul genotipului donor asupra succesului culturilor de microspori izolaţi a fost investigat de mai mulţi autori la diferite specii cultivate, rezultatele demonstrând că acesta joacă un rol cheie. Factorii genetici influenţează nu numai viabilitatea microsporelor şi frecvenţa embrionilor normali, ci şi rata de regenerare a plantelor. În această lucrare este prezentată o examinare completă a viabilităţii microsporelor, precum şi dezvoltarea embrionilor sub influenţa genotipului.*
Cuvinte cheie: haploizi, varza, *in vitro*, regenerare, plante

INTRODUCTION

Now-a-days, the microspore embryogenesis represents a unique method of single cell reprogramming in plants, through which a highly specialized cell, by specific stress treatment, switches its development towards an embryogenesis pathway.

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Numerous factors e.g., donor plant's genotype and physiology, microspore developmental stage, culture conditions and pretreatment affect the microspore embryogenesis (Tang *et al.,* 2003). Successful application of microspore culturing techniques for *in vitro* high-frequency plant regeneration largely depends on genotype, choice of explant, hormonal combination in the medium and other culture conditions.

The effect of donor genotype on the success of isolated microspores cultures was investigated by many authors in different cultivated species, the results demonstrate that it plays a key role. Genetic factors not only influence the viability of microspores and frequency of normal embryos but also the rate of plant regeneration (Cloutier *et al*, 1995).

The studies also underlined the existence of so-called recalcitrant genotypes (Wang, 2009), whose microspores although cultivated under growth conditions that proved to be inductive and beneficial for other genotypes fail to generate embryos.

Thus, at *B. napus* among the many genotypes tested 'Topas' variety was identified to be the most suitable, which is also taken as a model plant, being commonly used in research work aimed at improving cultivation techniques and technologies by modifying certain parameters (Ajisaka, 1999). Also, in other species of Brassica genotypes with increased capacity of the generation of embryos, as well as the recalcitrant genotypes were identified. For example, in *B. carinata* the 19 genotypes tested on the same environmental conditions led to a highly heterogeneous response rate of embryogenesis (Chuong *et al*., 1989).

In this paper, a complete screening of microspores viability as well as embryo development under the influence of genotype is presented.

MATERIAL AND METHOD

The mother plants are grown in 20 cm plastic pots, in greenhouses until the stage of 10 leaves. Afterwards the plants are vernalised for 90 days in growth chambers at 4^0C, in 16 h photoperiod conditions with active photosynthetic active radiation of almost 60 µmol m^{-2} s^{-1}, and than passed into the same 16 h photoperiod but with a temperature regime of 15^0C during light and 10^0C during dark. The plant fertigation was accomplished weekly with liquid fertiliser (N:P:K – 20:10:20).

Since one of the most important aspect for a succesful culture of microspores is the age of the donor plants, from our previous experiences we collected the biologic material from 12-14 week old plants. Thus, healthy floral buds of 3,2-3,5 mm represents the most effective biological material that can ensure a homogenous microspores population with high embriogenic competence. The excised buds were surface sterilized in 0.1% mercuric chloride (w/v) for 15 min, followed by rinsing in sterile distilled water for 3 to 4 times.

The microspore culture was initiated under sterile condition. The buds were squeezed gently with a piston taken from a 10 mL disposable syringe into a small glass vial, releasing the microspores in 10 mL NLN medium (Lichter, 1982) containing 13 g of sucrose. The suspension is filtered through a sterile 40 µm nylon mesh and the filtrate was centrifuged 3 minutes at 200 g. The supernatant is discarded and pellet is resuspended in 10 mL medium and centrifuged again. The procedure is repeated three times. Finally, the microspores are suspended in 1-2 mL

of NLN medium and plated in petri dishes with a density adjusted to $1x10^4$ microspores/mL. The cultures are subjected to a heat stress by incubating the cultures in darkness at 33^0C for three days. Following the same procedure, the renewal of the medium is accomplished after the heat shock and the culture plates are incubated to 25^0C in dark for three weeks.

For the screening of the microspore viability during the early period after inoculation, the FDA (fluorescein diacetate) staining squash method was utilised. Thus, 150-300 µl suspension with micropores is transferred in an Eppendorf tube. The volume is completed with culture medium until 1 mL and 1 mL stock solution of FDA medium is added. After 2 minutes the suspension is centrifugated and the pellet is removed. 10 µL of suspension is used for squash sampling and observed under UV filter microscope HUND 600. The bright green cells are recorded and utilised for statistical analysis. The number of viable microspores in different stages was counted in randomly selected visual areas of the microscope in four replications per sample.

The experiments were accomplished in three replications, each one containing five plates per variant. The viability of microspores and the mean number of embryo per variant was recorded. The data were analyzed by ANOVA (analysis of variance). The means were compared using the Duncan multiple comparison test at $P < 0.05$.

RESULTS AND DISCUSSIONS

The experimental work was aimed at carrying out a study of testing a wide range of germplasm - lines, varieties and hybrids in order to determine the androgenic capacity of germplasm resources, usable in breeding activities. Thus, 16 genotypes belonging to Vegetable Research and Development Station Bacau were tested:

- lines (BCC29, BC 145, BC 228, BC 341, CCA 429, CCA 440),
- varieties (ZM 12, ZM 131, ZM 202, ZM 321, ZM 323, ZM 622),
- hybrids (D14, H19, R10 şi T11).

The morphogenetic response of the microspores grown under the conditions described above varied significantly, closely dependent on the donor genotype. The amplitude of this response was found between 0 embryos/petri dishes in CCA 440 genotypes and 196 embryos/petri dishes at ZM12 genotype. Of the 16 genotypes tested, very good results were obtained for ZM 202, ZM 131 and hybrids D14, R10 and T11.

Thus, plasmolyzed microspores were identified (fig. 1), small micropores but which although viable have ceased their growth and development processes, microspores with polical evolution (fig. 2), as well as microspori who have acquired embryogenic skills (fig. 3), allowing the dihaploid embryos to be obtained.

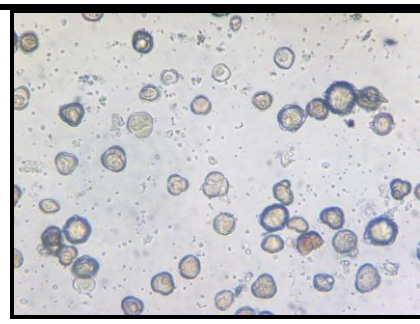

Fig. 1 Plasmolyzed microspors, genotip CCA 440 (foto stereomicroscop – 40x)

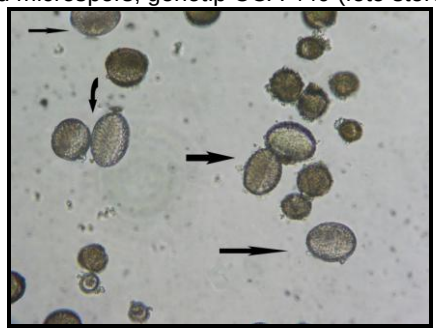

Fig. 2 Microspor with pollinic evolution (foto stereomicroscop 60x)

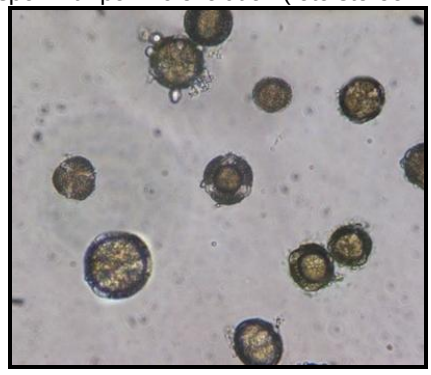

Fig. 3 Microspor with embriogenic competence (foto stereomicroscop 60x)

Genotypic differences were also identified with respect to the percentage of viable microspores and the time period as they maintain this viability, the results obtained being synthesized in table 1.

These results confirm the theory formulated by many authors (Baro, 1999; Wang, 2009) that there is a certain genetic predisposition towards embryogenesis, a predisposition that once identified can be used in the breeding work aimed toward the transfer of this feature to genotypes with high agronomic potential, but with poor reaction to *in vitro* cultivation conditions. The viability of microsporus, registered in our work varied and in terms of the percentage of viable microspors, the minimum

threshold of 20% having a strong applicative character, leading to the identification of the morphogenetic reaction orientation of the microspore culture from early stages. The best results were found for the genotypes ZM 12, ZM131, ZM 202, ZM 622, all of which are varieties, as well as hybrid genotypes R10 and T11.

Table 1

Evolution of microspores viability under the influence of donor plant genotype

Nr. crt	Genotype	10 days	20 days
1	BCC 29	++	+
2	BC 145	++	+
3	BC 228	++	++
4	BC 341	++	++
5	CCA 429	+	-
6	CCA 440	-	-
7	ZM 12	+++	+++
8	ZM 131	+++	+++
9	ZM 202	+++	+++
10	ZM 321	+++	++
11	ZM 323	+++	++
12	ZM 622	+++	+++
13	D14	+++	+++
14	H19	+++	++
15	R10	+++	+++
16	T11	+++	+++

+++ - more than 20% embryogenic microspors
++ - embryogenic cells and viable microspors
+ - embryogenic cells and not viable microspors
- - without embryogenic cells, plasmolyzed microspors

At the opposite end were genotypes BC 145, BCC 29, CCA 429 and CCA 440, consanguineous lines used in breeding works at *Brassica oleracea*. For these genotypes, the viability of the microspors had a downward curve, the values being below the 20% embryogenic microspor limit.

Regarding the evolution of embryogenic microspors, it was similar to all genotypes tested, identifying pro-embryonic structures with 2-4 cells, surrounded by exine, pro-embryos with suspensory-like structures, globular embryos, heart, the torpedo and the cotyledon stage. Not all embryos with microsporal origin had a normal evolution, some of them having different physiological deficiencies (eg anthocyanic or albinism) or morphological deficiencies (incompletely developed cotyledons, short or missing hippocotal axis, lack of root, etc.).

CONCLUSIONS

The morphogenetic response of the microspores grown under the conditions described above varied significantly, closely dependent on the donor genotype. Thus, the amplitude of this response was found between 0 embryos / petri dishes in CCA 440 genotypes and 196 embryos / petri dishes at ZM12 genotype. Of the 16 genotypes tested, very good results were obtained for ZM 202, ZM 131 and hybrids D14, R10 and T11.

These results confirm the theory formulated by many authors that there is a certain genetic predisposition towards embryogenesis, a predisposition that once identified can be used in the bredding work aimed toward the transfer of this feature to genotypes with high agronomic potential, but with poor reaction to in vitro cultivation conditions.

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