RESEARCHES REGARDING THE ANALYSIS OF THE FACTORS THAT INFLUENCE THE EMBRYOGENESIS IN MICROSPORE CULTURES OF BRASSICA NAPUS

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Abstract

In order to develop an efficient technique of microspore culture for oilseed rape, we studied the influence of the developmental stage of floral buds on the embryogenesis potential of *in vitro* culture of microspores. We observed that the floral buds size may be a good indicator of the microspores developmental stage. For this purpose we determined the mean number of nucleus per microspore in five genotypes of *Brassica napus* at different developmental stages of floral buds. This index was correlated with the microspores embryogenesis capacity cultivated on MS medium supplemented with 1 mg/l 2.4D and 1 mg/l BAP.

Key words: embryogenesis, microspores, in vitro culture.

The double haploid plants are usually obtained by androgenesis and they are an efficient tool for producing homozygous lines in breeding programs. The microspore cultures lead to a significant time economy in breeding processes because double haploid plants can be obtained in 9 months, while in the classical breeding methods this can last at least 2 to 6 years. The double haploid plants are obtained *in vitro*, starting from the haploid cells of the microspores, anthers or ovules (Forster et al. 2007; Kott & Beversdorf 1990). Generally, the microspore culture is the most used method for obtaining double homozygous cultivars because it has a better efficiency and excludes the development of the somatic embryos from another source such as the diploid tissues of the anther. The microspore culture is based on the possibility of switching the maturation process of the microspores, represented by the immature pollen grains, towards embryogenesis induction (Ilic-Grubor et al. 1998; Maraschin et al. 2005). Although the advantages of using this technique are obvious, the success of its application on a large scale depends on the possibility to determine the factors that could influence the embryogenesis process. There are various factors that influence this process and they have been described in the specialized literature (Ferrie et al. 1995; Sopory and Munshi 1996; Wang et al. 2000; Touraev et al. 2001).

The success of the microspores culture is influenced by the genotype and the cultivation conditions of the donor plants. The developmental stage of the microspores is also a very important trait for the success of the culture. Regarding this theme, some authors reported that the optimum developmental stage of the micronucleus to generate embryos is the late mono nucleate phase to the early bi nucleate phase. However, this stage is very difficult to be detected by cytogenetic methods. There is also a significant correlation between the morphology of the flower buds and the maturation stage of the microspores. The aim of this study was to determine the average number of nuclei for different sizes of the flower buds in five *Brassica napus* cultivars, which are going to be the starting material of the microspore culture, in order to determine the optimum micronucleus stage for a maximum microspore embryogenesis response.

MATERIAL AND METHOD

a) Cytogenetic studies

For determining the average number of micronuclei, flower buds of different sizes (2-8 mm) were harvested and kept in freezer at -80^{0}C. From every flower bud, anthers were collected. By squash method, microscopic probes were made by staining the anther with acetic-carmine dye in order to get the microspores out of the anther and to examine the nucleus stage of the microspores. For each probe there were visualized an approximate number of 400 microspores. The probe was

repeated three times for every bud size. We calculated the average number of nuclei/cell.

b) Microspore culture

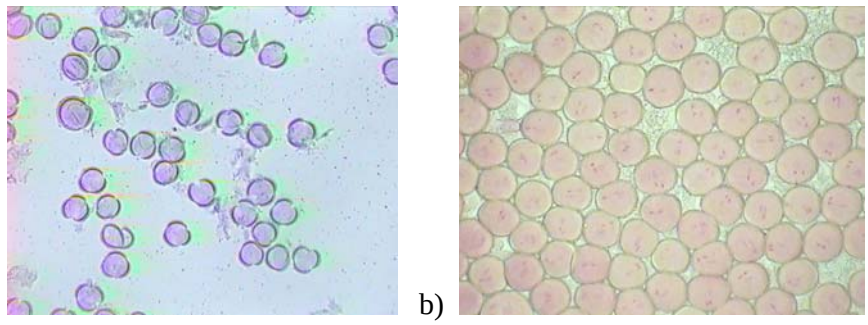
The donor plants were five oilseed rape cultivars (Lirajet, Maras, Elena, Glacier and Heimer) which were gown in normal field conditions. At the physiological time of flowering, healthy buds of different sizes (mostly 2-8 mm) were collected and kept in the freezer. For microspore isolation, 6-8 inflorescences were taken and desired buds (mostly 3- 8 mm) were picked with a fine tweeze and classified in 4 lengths group. Ten buds per length group were selected. The buds were sterilized for 10 minutes in 2% sodium hypochlorite.

The buds were rinsed in sterile tap water for 1, 4 and 10 minutes. The buds were homogenized by adding 2 ml of sterile MS (Murashige and Skoog) medium in a 50 ml beaker. Rinsing the piston of the 50 ml beaker, the suspension was poured over the sterile filter funnel with 10 ml centrifuge tubes. The volume of all the tubes was adjusted up to 10 ml. The microspores suspension was centrifuged 3 minutes at 800 rpm. Supernatant was poured off and the pellet has been resuspended with l0 ml medium. Centrifugation and resuspension was repeated twice. After the last wash, microspores were resuspended in 1 ml MS medium. The microspore suspension was poured in 15 cm diameter Petri dishes in MS liquid medium supplemented with 1 mg/l 2,4 D and kinetin and tapped with Para film. The Petri dishes were placed at 35°C for 2 days and then transferred to 25°C continuously in dark. The embryos development was observed by a optical microscope. After 14 days, globular embryo structures were found.

Table 1

Size of the flower buds and number of nuclei

Genotype	Number of repeats	2-3 mm	3-4 mm	4-5 mm	5-6 mm	6-7mm	>7mm
Lirajet	average	1	1.03	1.23	1.70	1.97	2
	1	1	1.1	1.3	1.6	2	2
	2	1	1.0	1.2	1.7	1.9	2
	3	1	1.0	1.2	1.8	2	2
Maras	average	1	1.13	1.27	1.70	1.90	2
	1	1	1.2	1.2	1.9	2	2
	2	1	1.1	1.3	1.6	1.8	2
	3	1	1.1	1.3	1.6	1.9	2
Elena	average	1	1.00	1.33	1.77	2	2
	1	1	1.0	1.3	1.8	2	2
	2	1	1.0	1.4	1.7	2	2
	3	1	1.0	1.3	1.8	2	2
Glacier	average	1	1.07	1.17	1.70	1.97	2
	1	1	1.1	1.1	1.6	2	2
	2	1	1.0	1.2	1.8	1.9	2
	3	1	1.1	1.2	1.7	2	2
Heimer	average	1	1	1.13	1.53	1.93	2
	1	1	1.0	1.1	1.7	2	2
	2	1	1.0	1.2	1.5	2	2
	3	1	1.0	1.1	1.4	1.8	2



a) b)

Fig. 1 Aspects of the microspores stages

a) The tetrad stage of the micronuclei from the flower buds with size less than 3 mm; b) The mean number of nuclei of the microspores harvested from different oilseed rape genotypes

Table 2

Number of microspores in the division phase

Genotype	Number of repeats	3-4 mm	4-5 mm	5-6 mm	6-7mm
Lirajet	1	15	51	21	0
	2	10	73	16	3
	3	13	45	9	0
	average	12.67	56.30	15.33	1.00
Maras	1	16	55	10	0
	2	8	43	12	0
	3	9	41	15	0
	average	11.00	46.33	12.33	0.00
Elena	1	12	67	13	5
	2	7	52	10	3
	3	11	49	9	1
	average	10.00	56.00	10.66	3.00
Glacier	1	13	35	7	0
	2	9	47	13	2
	3	6	40	13	0
	average	9.33	40.66	11.00	0.67
Heimer	1	5	15	2	1
	2	2	30	4	1
	3	7	27	5	2
	average	4.67	24.00	3.67	1.33

RESULTS AND DISCUSSIONS

One of the crucial factors of the embryogenesis is the establishment of the developmental stage of the microspores. The cytogenetically studies made by several authors show that the optimum moment for the microspore isolation in order to induce embryogenesis is the end of the mono nucleate phase and the beginning of bi nucleate stage. Because the use of cytogenetically techniques in determining the optimum stage of microspore development is difficult to apply, in this study, we aimed to determine the average number of nuclei for microspore isolated from flower buds of different sizes, in order to establish a correlation between the flower bud size and the developmental stage of the microspores.

This could be an easier technique for determining the optimum moment for harvesting the flower buds for the microspores, in order to develop an embryogenesis process. The first table shows the values obtained in this study. It can be observed that for the flower buds with 3-7 mm in size, the average number is situated between 1 and 2 that corresponds to the mono and bi nucleate stages of the microspores presented by the other authors as the optimum stage for the isolation of microspores. The cytogenetic exam of the flower buds smaller than 3 mm revealed that the microspores were in the mono nucleate stage. Most of them were in the tetrad stage as shown in figure 1 – a, not yet transformed in nuclei. Although it has been recorded a variation for this trait from one individual to another, and from one genotype to another (*fig. 2b*), we can tell that the size of the flower buds can be a quite good indicator for determining the stage of the microspore in *Brassica napus* species.

For the next stage of the experiment, we eliminated the flower buds smaller than 3 mm and bigger than 7 mm using four types of flower buds sizes presented in Table 2, which were used as a starting material for the microspore culture. For this, we used the MD medium (Murashige and Skoog) supplemented with 1mg/l 2,4D and 1 mg/l K and the samples were incubated at 25^0C in dark. After 2 weeks, we determined the number of microspores in division stage, in order to establish the optimum nuclear index which reveals the perfect size of the flower buds capable of embryogenesis. Analyzing the obtained data, it was observed that for the *Brassica napus* species, the optimum size of the buds in order to generate embryos is 3-7 mm. Still, the best results were obtained for the microspores with a nuclear index of 1,3 and the flower bud size of 4-5 mm. The embryo number that gives an embryo answer was 400% higher than the one obtained in the variant where the starting material was represented by flower buds of other sizes. This data confirms the results obtained by other authors (Telmer et al., 1992, Vincente and Dias, 1996), who consider that there is a positive correlation between the size of the flower buds and the developmental stage of the pollen. On the other hand, other authors consider that only the microspores that are at the end of the mono nucleate stage and the beginning of the bi nucleate stage can generate embryos (Pechan and Keller 1988, Hansen and Svinnset 1993), but those

results are not confirmed yet. In this experiment we detected higher differences between the studied genotypes, but the optimum size of the flower buds and the nuclear indices are the same independent of the studied genotype.

CONCLUSIONS

Analyzing the dates, we can conclude that:

The average number of nuclei is a quantitative indicator which reflects the developmental stage of the microspores and it is a sensible index for establishing the optimum moment for harvesting the flower buds for microspore cultures.

In *Brassica napus* species the optimum stage for harvesting the flower buds for the adrogenesis process is when the mean number of nuclei from microspores is between 1,2 and 1,5 and the size buds is constant (4-5mm) for all the genotypes taken in study.

Although the morphogenetic response of the five studied genotypes is different, still the optimum size of the flower buds is the same (4-5 mm) and the use of the nuclei in this developmental stage can increase 500 % the frequency of the embryo structure appearance at this species.

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