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**ORIGINAL PAPER**

# EFFECT OF CARBOHYDRATE TYPE OVER THE MICROSPORE EMBRYOGENESIS AT *BRASSICA OLERACEA L.*

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## Abstract

*Microspore culture triggers the attention of specialists all over the world, due to its importance in obtaining double haploid plants, a true homozygous line that can used in breeding for the obtaining of $F_1$ hybrids, shortening the period in which they can be obtained with at least 6-7 years. The ability to change from the normal gametophytic development of pollen to a sporophytic one, finalized with haploid embryo formation and ultimately with double haploid plants by spontaneous or induced diploidization is conditioned by many intrinsic and extrinsic factors.*

*Among the many factors that affect the viability and the capacity of microspores cultivated on liquid mediums in vitro to switch their developmental programs from gametophytic way to the sporohytic one, finalized with the obtaining of double haploid embryos and plants, the carbohydrates play a critical role. They act both as a source of energy for the growth and development of cells and as a osmoticum. To investigate the effects of different carbon sources on embryo induction in liquid cultivation system, microspores were cultured in NLNS medium containing 6, 9, or 12% (all w/v) of sucrose, fructose, glucose and maltose.*

**Keywords**: embryo, glucose, haploid, maltose, sucrose.

## Introduction

The microspores are highly specialized cells that exposed to certain environmental conditions as well as various stress, can switch their normal gametophytic evolution toward embryogenesis leading to the obtaining of double haploid plants. Keeping in mind the fact that at *Brassica* species, for the obtaining of homozygosity plant, breeders usually needs 8-10 year to achieve it by self-pollination, the obtaining of microspore-derived embryos, that are double haploids, within one generation provide a powerful tool for the optimization of these processes. The importance of microspore embryogenesis is highlighted by its wide utilization in other scientific approaches relevant for crop improvement: various genetic manipulations, transformation, gene expression, gene mapping, etc. Since the first success of obtaining haploid plants from microspore-derived embryos, achieved by Lichter, 1982, at *Brassica napus* L., the effort of the specialists were focused toward the development of protocols for the cultivation of microspores in other members of *Brassica* genus, such as: *B. juncea* (Canana, 2005; Prem et al., 2009), *B. carinata* (Abraha et al., 2008; Wang, 2009), *B. oleracea* var. *intalica* (Lemonnier-Le Penhuizic, 2001), *Brassica oleracea* var. *acephala* (Zhang, 2008), *B. campestris* (Wang, 2009). Due to the fact that these species are rather recalcitrant to microspore in vitro manipulation, this feature being highly dependent to exogenous (medium conditions, controlled applied stresses) and endogenous factors (genotype dependency), the researchers are in a continuous search of improving the existing protocols as well as to adapt it to specific genotypes requirements.

As mentioned before, the efficiency of microspore culture is affected by a number of factors, including the growth conditions of the donor plants, the microspores developmental stage, the microspore plating density, the type of stress treatment applied, the culture medium composition, etc. Among them the availability of carbon in culture medium, dependent on the type of carbohydrate utilized play an important role. The carbohydrate is a major component of culture medium because it provides the energy necessary for growth and development processes of microspores and also act as an osmotic regulator. The most frequent type of carbohydrate is sucrose. The studies publishes results in which the embryo yield enhances in the presence of maltose, glucose, fructose or mannitol as carbon source.

Since the ability of microspores to undergo divisions and generate DH plants has a strong genotype dependency, for the determination of viable culture protocol of microspores a complete screening of the morphogenetic reaction of microspores under the influence of carbohydrate type and concentration was accomplished. The results presented in this study are part of a larger study focused toward the establishment of a sustainable protocol for the cultivation of local *B. oleracea* genotype microspores and ultimately for the obtaining of isogenic lines utilizable as parent plants in hybridization.

## Materials and Methods

*Plant growth conditions and biologic material*

The donor plants were provided by Vegetable Research and Development Station Bacau. 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 $4^0$C, 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 $15^0$C during light and $10^0$C during dark. The plant fertigation was accomplished weekly with liquid fertiliser (N:P:K – 20:10:20).

The biologic material, collected from 12-14 week old plants is represented through healthy floral buds of 3,2-3,5 mm, containing microspores at uninucleat stage, as determined in our previous studies (data unpublished) to be the most effective dimension for the obtaining of 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.

For the accomplishment of the screening of carbohydrate type and concentration influence over the ability of microspores to generate DH embryo 12 variants of culture medium were tested – table 1.

**Table 1.** Experimental variants tested during 2011-2012 for the determination of most beneficial type and concentration of carbohydrates for DH embryo formation

| No. crt. | Variant | Type | Concentration % |
|---|---|---|---|
| 1. | V1 | sucrose | 6 |
| 2. | V2 | | 13 |
| 3. | V3 | | 20 |
| 4. | V4 | glucose | 6 |
| 5. | V5 | | 13 |
| 6. | V6 | | 20 |
| 7. | V7 | fructose | 6 |
| 8. | V8 | | 13 |
| 9. | V9 | | 20 |

| 10. | V10 | maltose | 6 |
|---|---|---|---|
| 11. | V11 | | 13 |
| 12 | V12 | | 20 |

*Microspore culture*

Under sterile condition the buds are 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 $33^0$C 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 $25^0$C in dark for three weeks.

*Plant regeneration*

After 4 weeks of culture, each normal cotyledonary embryo (with well developed radicle, hypocotyl and two symmetrical cotyledons) was transferred to regeneration medium B5 (Gamborg 1968) supplemented with PGRs - BAP- 8.9 μM and NAA 2.7 μM, 2 % sucrose and 0.08% agar-agar. The cultures were grown in a growth chamber at $25^0$C under a 16/8 h (day/night) photoperiod. Eight weeks after embryo transfer to the regeneration medium, plantlets with 2–5 leaves were transplanted to soil and the pots were covered with transparent vinyl for 2 weeks to allow acclimatization. The potted plantlets were grown in a growth room under the conditions described above for the flower-bud donor plants.

*Cytological studies*

For the determination of the medium influence over the orientation of developmental processes of *Brassica oleracea* microspores cultivated in vitro we utilised the FDA (fluorescein diacetate) staining squash method for the screening of their viability during the early period after incoulation. 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.

*Statistical analysis*

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 Discussion

The results obtained in the present study reveals the fact that although sucrose seemed to be the most suitable source of carbohydrate (Abraha, 2008), maltose can also promote the development of uninucleated microspores toward embryogenesis. Moreover, the number of medium renewal is strongly reduced as the microspores maintain their viability and continue their growth and development without the need of transfer to new fresh medium. This situation is correlated with the fact that maltose is more slowly metabolized than sucrose, thus

existing enough available oxygen in the culture. On mediums with glucose, fructose and even sucrose, the microspores rapidly metabolize the sugars, they became hypotoxics, accumulating a large quantity of ethanol in their cells, arresting the growth and ultimately loose their viability.

In sucrose containing mediums the viability of microspores decreases rapidly during the first days of culture, renewal of the culture medium being the only effective way to control this lost. If after inoculation about 80-85% of the microspores were viable – fig. 1, the percentages gradually decreased until 21,6% after 10 days and 12,9% after 20 days in sucrose containing mediums, respectively 49,7% after 10 days, and 28,8% after 20 days in maltose containing mediums.

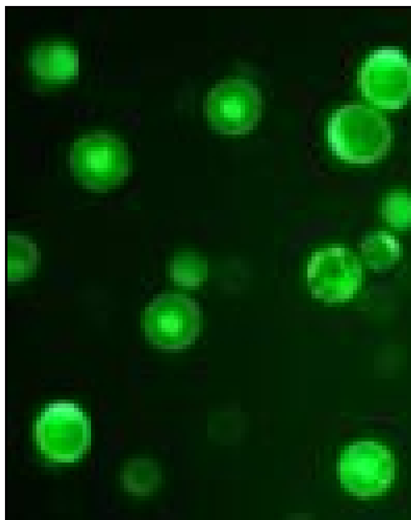

**Fig. 1** – Viable microspores, FDA staining

When the carbohydrate source was glucose or fructose, the results obtained were even lower, under 20% (assigned as reference point) and although the microspores were transferred to fresh mediums no markedly improvement has been registered. The results are synthesized in table 2.

**Table 2.** The microspore viability 10 and 20 days after inoculation on NLN medium

| Variant | Concentration (%) | Type of carbohydrate | After 10 days | After 20 days |
|---|---|---|---|---|
| V1 | 6 | sucrose | +++ | ++ |
| V2 | 13 | | +++ | ++ |
| V3 | 20 | | ++ | + |
| V4 | 6 | glucose | + | + |
| V5 | 13 | | ++ | ++ |
| V6 | 20 | | - | - |
| V7 | 6 | fructose | - | - |
| V8 | 13 | | - | - |
| V9 | 20 | | - | - |
| V10 | 6 | maltose | +++ | +++ |
| V11 | 13 | | +++ | +++ |
| V12 | 20 | | +++ | +++ |

+++ more than 10% embryogenic microspores;
++ embryogenic cells and alive microspores;
+ embryogenic cells and dead microspores,
- no embryogenic cells, plasmolised microspores.

The results obtained in the present study revealed that fructose does not sustain the embryogenic development of microspores, the viable microspores presenting a strong accumulation of starch grains in their cells, process that is characteristic for the gametophytic development of the microspores. Glucose, although benefic in other species Triticalae (Touraev, 1996), cotton (Herlebe-Bors, 1999) is also not efficient for the sustainability of viability and embryogenesis of *Brassica oleracea* microspores. On these variants the microspores increases rapidly in size, accumulates starch and arrests their growth, resembling as gametophytic-like cells while the embryogenic microspores became swollen and started to divide in two nuclei after the first 2-3 days of culture.

In fructose and glucose containing medium these accumulations of starch gradually led to cell lysis, more pronounced in the case of fructose while sucrose-containing medium, although initially with the same trend of accumulation of starch, the repeated transfer on fresh culture media helped to overcome these impediments. Moreover, the experiments conducted have revealed the fact that, in the case of media with sucrose importance for the orientation process of gametogenesis and embryogenesis is the rapid transfer of microspores (3 days after inoculation) on fresh medium, an operation which seems to be a effectively way to overcome the problems caused by the accumulation of toxic products of metabolism, mainly higher in the early inoculation, most likely caused by the shock isolation. For media with maltose the transfer on fresh medium after 3 days did not positively influence the processes of embryogenesis and the development of microspores is comparable to the variants with sucrose in which the transfer of microspores on fresh medium was accomplished. These results underline the effectiveness of maltose-containing medium as the same results can be obtained while avoiding time consuming transfer, enhancing the cost efficiency of microspore culture protocol.

On sucrose and maltose containing mediums the cell population was highly asynchronous during the first ten days – fig. 2, containing small dead microspores, gametophytic–like micropores star-like microspores – fig. 3 and two-three cell pro-embryo – fig. 4.

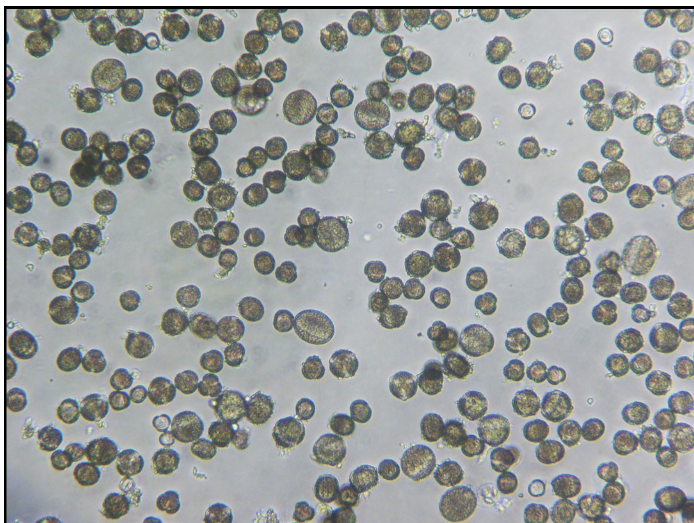

**Fig. 2** – Asynchronous population of microspores, 24 hours from inoculation (40x)

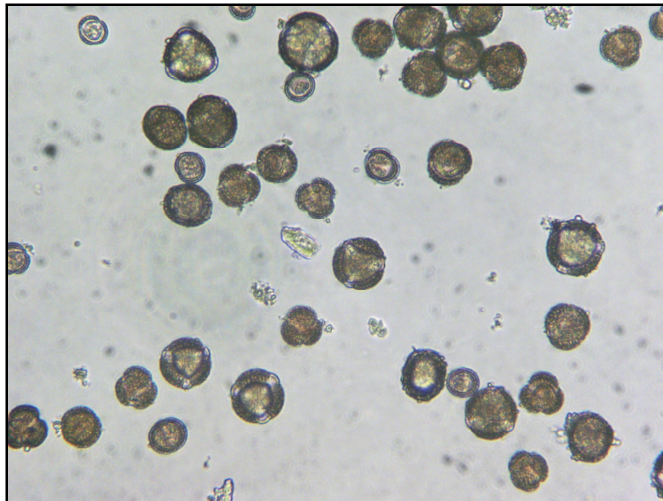

**Fig. 3** – Microspores with embryogenic competence on maltose-containing medium (60x)

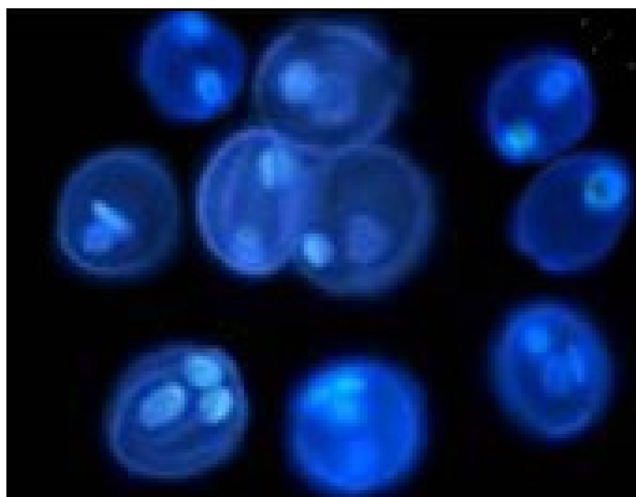

**Fig. 4** – DAPI stained microspores showing two or three nuclei

After further development, the two nuclei divided giving raise to globular proembryos – fig. 5.

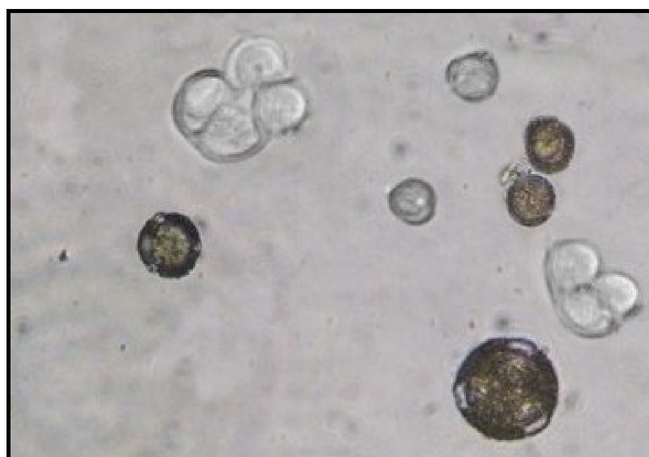

**Fig. 5** – Pro-embryonary structures with three or four cells (100x)

As shown in table 3, in the case of cultures with fructose or glucose containing media, however, embryos were not detected at 3 weeks after culture, and only a small number of tiny globular embryos appeared at 4 weeks after culture.

**Table 3.** The effect of carbohydrate type and concentration over the induction and development of embryo in isolated microspore culture at *Brassica oleracea L.*

| No. crt. | Variant | Carbohydrate type | Concentration | Average number of embryo/Petri dish |
|---|---|---|---|---|
| 1. | V1 | sucrose | 6 | 20,0±2,2 |
| 2. | V2 | | 13 | 39,0±3,6 |
| 3. | V3 | | 20 | 28,0±2,9 |
| 4. | V4 | glucose | 6 | 5,2±0,8 |
| 5. | V5 | | 13 | 5,8±1,3 |
| 6. | V6 | | 20 | 2,2±0,7 |
| 7. | V7 | fructose | 6 | 1,2±0,8 |
| 8. | V8 | | 13 | - |
| 9. | V9 | | 20 | - |
| 10. | V10 | maltose | 6 | 30,4±3,8 |
| 11. | V11 | | 13 | 45,4±6,5 |
| 12 | V12 | | 20 | 37,5±0,5 |

One of the goals of the present study was to investigate the effect of sugar concentration - 6, 13 and 20% (w/v) on the induction and development of embryos in microspore culture of white cabbage.

The average number of embryos that developed in sucrose-supplemented medium was 39 embryos per Petri dish, while microspores growing in maltose-supplemented medium yielded 45.4 embryos. The results showed that the maximum number of cotyledonary embryos was obtained when the microspores where inoculated on 13% maltose-containing medium, respectively 45,4±6,5 embryos/plate, while at lower concentrations only 30,4±3,8 embryos normally developed – table 3. This showed that maltose, was more effective than sucrose, as a carbon source for embryonic induction and development, with the most suitable concentration of 13% (w/v). Our results are consistent with those reported by previous authors which stated that the addition of maltose efficiently induced microspore embryogenesis, and increased green plant regeneration in cereal species (Scott, 1995), potato (Dunwell, 2010), pepper (Supena, 2006) or those obtained with *Brassica napus* and *Brassica oleracea* protoplast (Loudon, 1989). Scott et al., 1995 suggested that the microspores thrive on maltose containing medium due to the fact that the metabolism of maltose is slower and there is sufficient oxygen available to allow cells to survive on culture.

After five weeks in culture, the capacity of microspores to generate and develop into embryo declined, moreover, the already formed embryos at globular and heart-shaped stage, or abnormal embryos began to undergo necrosis, probably because of nutrient depletion. When normal cotyledonary embryos were transferred to solid B5 (Gamborg 1968) supplemented with PGRs - BAP- 8.9 μM and NAA 2.7 μM, 2 % sucrose and 0.08% agar-agar, and held in growth chamber at $25^0$C under a 16/8 h (day/night) photoperiod, they turned green, initiated active growth and within eight weeks developed into fully acclimatized plants.

## Conclusions

In order to investigate whether the type of carbohydrate used as a carbon source might affect the efficiency of embryogenesis, a complete screening of the main types of

carbohydrates in different concentrations were tested. Both microspore viability and embryo induction and development were examined, each variant was repeated three times, with six replicates. The results obtained show that maltose, rather than sucrose is the most suitable carbohydrate source with the most effective concentration of maltose of 13% (w/v), functioning optimally as both an osmotic regulator and a carbon source for *Brassica oleracea* microspores.

## Acknowledgements

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