ANALYSIS OF CULTURE MEDIUM INFLUENCE OVER THE MICROSPORE EMBRYOGENESIS AT *BRASSICA OLERACEA* L.

ANALIZA INFLUENȚEI MEDIULUI DE CULTURĂ ASUPRA EMBRIOGENEZEI MICROSPORILOR DE *BRASSICA OLERACEA* L.

CRISTEA Tina Oana[1], LEONTE C.[2], PRISECARU Maria[3], AMBĂRUȘ Silvica[1], CĂLIN Maria[1], BREZEANU Creola[1], BREZEANU P.M. [1]
e-mail: sclbac@legumebac.ro

Abstract: *The culture of isolated micropores offers the opportunity to generate double haploid embryos starting from single haploid cells, thus assuring the genetic purity of haploid plants obtained. For Brassica species double haploid plants are strongly required for use as isogene lines in F1 hybrids production. By this innovative technique the time required is dramatically shortened from 8-9 years to one year generation. The advantages brought by its utilization tagger the interest of specialists to improve the culture conditions in order to assure the efficiency of the culture, as the species from Brassica genus are recalcitrant to cultivation, displaying a strong genotype dependency. In this study three main type of culture medium formulation were tested and the viability of micropores as well as embryo development were recorded in order to achieve an efficient protocol in Brassica oleracea Romanian genotypes.*
Keywords*: haploids, embryo, somatic, cabbage, viability*

Rezumat: *Cultura de microspori izolati ofera oportunitatea generarii de embrioni dublu haploizi pornind de la o singura celula haploida, astfel asigurand puritatea genetica a plantelor obtinute. Pentru genul Brassica plantele dublu haploide sunt extrem de importante din perspectiva utilizarii lor ca linii izogene in procesul de generare a hibrizilor F1. Prin intermediul acestei tehnici inovative timpul necesar producerii acestor linii se scurteaza considerabil de la 8 - 9 ani, cat e necesar in conditii de ameliorare clasica la un an. Avantajele aduse de utilizarea acestei tehnici a determinat cresterea interesului specialistilor in imbunatatirea conditiilor de cultura pentru a asigura eficienta acesteia, stiut fiind faptul ca o parte din speciile genului Brassica sunt reclacitrante fata de acest tip de tehnica de cultura, sustenabilitatea ei fiind in stransa dependenta de genotip.*
Cuvinte cheie: *haploizi, embrion, somatic, varza, viabilitatea*

INTRODUCTION

Doubled haploid (DH) plants have been used in breeding programs for many years to develop improved crop varieties (Thomas et al., 2003, Ferrie and Mollers, 2011). The production of haploid plant *in vitro* is more efficient than

[1] Vegetable Research and Development Station of Bacau, Romania
[2] University of Agricultural Sciences and Veterinary Medicine of Iasi, Romania
[3] „Vasile Alecsandri" University of Bacau, Romania

conventional plant breeding for the generation of diploid homozygous pure lines, because such lines are obtained in a single generation *in vitro*, while several generations of inbreeding are required using traditional means. One of the most common method for producing haploid plants has been the cultivation of whole anther *in vitro*. The use of isolated microspores, however, rather than the entire anther, is now considered the most efficient approach for haploid plant production, because the isolated microspore culture avoids the formation of calli and embryos from the somatic tissues of the anther. All embryos yielded from isolated microspore cultures can only be microspore-derived, thus assuring the certainty of being either haploids or doubled haploids. Another advantage offered by this innovative technique is the fact that it allows the direct access to the microspores, which speeds up the optimization of culture conditions, as all modifications are directly subjected to single cells. Third, isolated microspore culture produces a higher number of embryos than does anther culture.

In order for doubled haploidy to be effective in a breeding program, an efficient microspore culture protocol is required. The conditions leading to the induction and development of microspore-derived embryos vary depending on the species, and therefore doubled haploidy methods have to be determined for each species. A number of factors influence microspore embryogenesis including genotype, stage of microspore development, donor plant growing conditions, media composition, and culture conditions.

Regarding the culture media, species-specific differences in the efficiencies of embryogenesis and embryonic development from microspores are seen with various culture media. For example, the embryogenesis in wheat is more effectively induced using A2 (Touraev et al., 1996) or AMC media (Kunz et al., 2000), while in Brassica species the embryogenesis is efficiently induced with NLN or modified NLN media (Swanson, 1989), B5 medium (Gamborg et al., 1968) or MS (Murashige Skoog, 1962). Lichter (1982) first reported the successful application of microspore culture techniques to Brassica napus, thereby establishing the basis of isolated microspore cultures for the Brassica genus. Subsequently, successful microspore culture in different cauliflower (*Brassica oleracea* var. *botrytis*), broccoli (*Brassica oleracea* var. *italica*), tronchuda cabbages (*Brassica oleracea* var. *costata*), kohlrabi (*Brassica oleracea* var. *gongylodes*), ornamental kale (*Brassica oleracea* var. *acephala*). However, there are relatively few reports on microspore cultures of white cabbage (Yuan et al., 2012).

MATERIAL AND METHOD

Plant material

The open-pollinated variety DM 56 provided by Vegetable Research and Development Station Bacau was used in our study as donor plants. 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).

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.

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 medium 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 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. After that the embryo yield is determined and the cotyledonary embryos are transferred to B5 (Gamborg et al., 1968) solid medium for regeneration.

The variants tested in the present study are represented by basic medium formulas established by: variant V1 - NLN (Lichter, 1982), variant V2 - B5 (Gamborg, 1968), variant V3 – MS (Murashige Skoog, 1962).

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 DISCUSSIONS

Isolated microspores of flowering plants can undergo embryogenesis when cultured *in vitro* under appropriate conditions. To investigate the effect of culture medium over the orientation of morphogenesis of *Brassica* microspores we tested

three variants of the medium formulation: variant V1 - NLN (Lichter, 1982), variant V2 - B5 (Gamborg, 1968), variant V3 – MS (Murashige Skoog, 1962).

After the initiation of cultures the microscopic analysis revealed that more than 85% of microspores were viable – fig. 1.

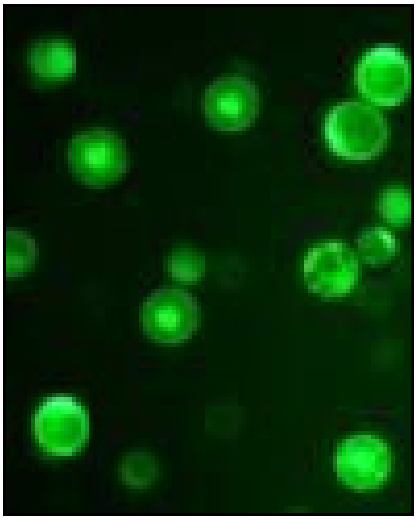

Fig. 1 – Viable microspores, variant V1- under fluorescence micropore 40x

The percentage decreased with culture time, as part of the microspores started to divide and became pro-embryos while a part of them were viable but showing no sign of embryogenesis, resembling more to gametophytic-like cells. The results registered in day 7 and 14 are synthetised in table 1.

Table 1

The evolution of microspore viability after 7 and 14 days of cultivation

Genotype	Variant	After 7 days	After 14 days
DL 20	V1	+++	+++
	V2	+++	+++
	V3	++	-

+++ - more than 10% embryogenic microspores
++ - embryogenic cells and viable microspores
+ - embryogenic cells and non-viable microspores
- - without embryogenic cells, dead microspores

As illustrated in the previous table, the optimum medium for sustainability of microspores viability, as well as for the switch of developmental processes toward embryogenesis is medium NLN – variant V1. After their transfer in new fresh medium, the evolution of the microspores was oriented toward the apparition of star-like microspores – fig. 2 and symmetric divisions.

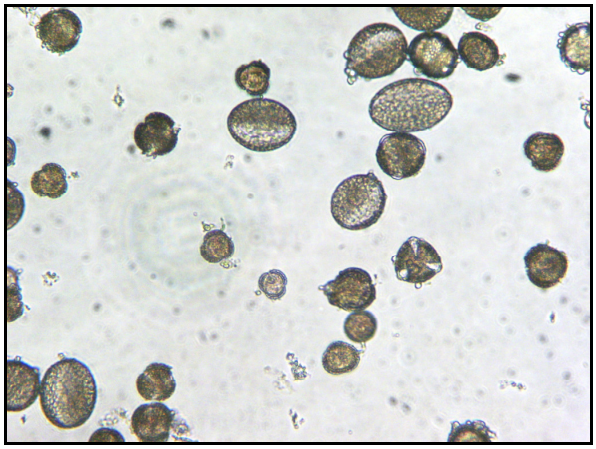

Fig. 2 – Heterogenous population of microspores with star-like microspores and gametophytic-like microspores

Thus the embryogenic microspores passed through a series of transformations of cellular organization, from two cells surrounded by microspore cell, exine to proembryos and globular, torpedo and cotyledonary embryos. During experimentation the embryo yeld was recorded, the results being presented as the average of the three replication for each variant – fig. 3.

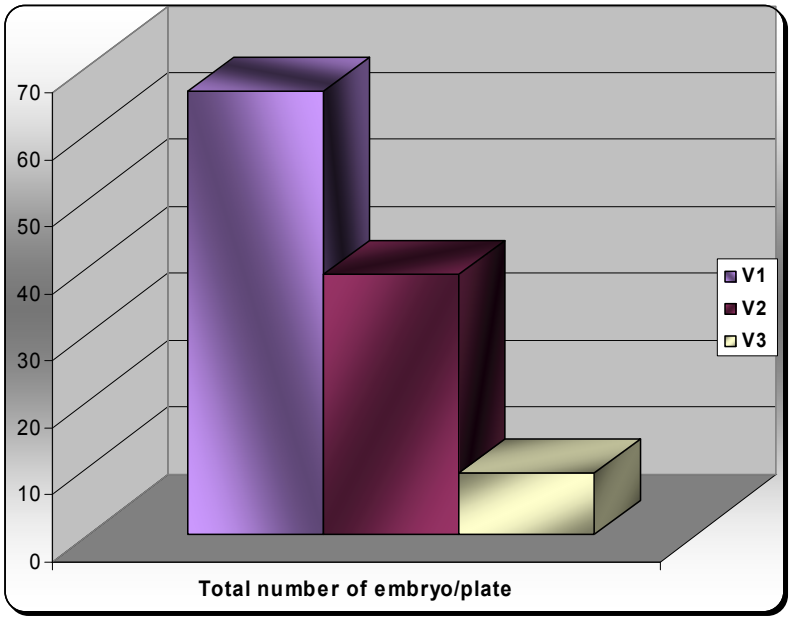

Fig. 3 – Graphical representation of frequency of embryo formation on different variants of basal culture medium

The highest frequency in embryo formation was recorded on NLN medium. From the total number of 114 embryo yielded from all variants 34 of them displayed different degree of abnormality: lack of cotyledons, albinism and slow

growth and died. The transfer of the normal embryos on B5 solid medium allowed their development in green plants and only 17 died during acclimatization. The acclimatized plants were potted individually and transferred to greenhouse and allowed to grow to maturity.

CONCLUSIONS

In this study three main type of culture medium formulation were tested and the viability of microspores as well as embryo development were recorded in order to achieve an efficient protocol in *Brassica oleracea* Romanian genotypes. The highest frequency in embryo formation was recorded on NLN medium, while the lowest results were obtained on MS medium. The transfer of the normal embryos on B5 solid medium allowed their development in green plants and only 17 died during acclimatization. The acclimatized plants were potted individually and transferred to greenhouse and allowed to grow to maturity.

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