AcademicPres

Available online at www.notulaebiologicae.ro

Print ISSN 2067-3205; Electronic 2067-3264

Not Sci Biol, 2013, 5(4):485-489

Notulae Scientia Biologicae

The Influence of pH on Microspore Embryogenesis of White Cabbage (*Brassica oleracea* L.)

Tina Oana CRISTEA*

Vegetable Research and Development Station Bacau,
220 Calea Barladului, Bacau, Romania; tinaoana@yahoo.com

Abstract

In vitro microspore culture is one of the top techniques utilised now-a-days for the obtaining of double haploid plants in many plant species, including *Brassica.* The pH of the medium is a critical factor for the success of *In vitro* microspore culture as it influences the invertase enzyme activity, translated at cellular level through an acceleration or reduction of sucrose cleavage. The results published until now shows rather contradictory findings, as the response of microspores have been proved to be highly depending on genotypes, most of them being focused on *Brassica napus*. Thus, in the present study, the effect of different NLN liquid medium pH, ranging between 5.0 to 7.0 were tested in order to establish the most suitable pH for the expression of embryogenic competences of microspores cultivated on medium *In vitro* and ultimately for the obtaining of microspore-derived embryos. Among the 11 values of pH tested, the best results were obtained on variants with pH 5.8 and 6.0, both in what concern the maintaining of microspores viability and the number of microspore-derived embryos. The findings of the present study provide a strong base for the establishment of an efficient protocol for the *In vitro* culture of microspore at *Brassica oleracea* L. genotypes with Romanian origin.

Keywords: embryo, haploid, protocol, regeneration, viability

Introduction

Brassica oleracea is an important vegetable plant, grown on large surfaces worldwide, occupying the third place in the world, after Solanaceae and onion. Most of the cultivars grown are represented by F_1 hybrids, superior to varieties due to heterosis, translated in higher uniformity, resistance and production, better protection of property-rights, etc. For the production of F_1 hybrids, homozygous plants, isogenic lines are required. If traditionally, plant breeders usually achieve homozygosity of the cross products through self-fertilization in 6-8 years, by microspore culture, homozygous plants are obtained within a year because such lines are obtained in a single generation *In vitro*, while several generations of inbreeding are required using traditional means. Even with 6 years of self pollination in a conventional plant breeding program, the breeder can only achieve about 98% homozygosity, compared to 100% with doubled haploidy techniques (Ahmadi, 2012). In order for doubled haploidy to be effective in a breeding program, an efficient microspore culture protocol is required. A number of factors influence microspore embryogenesis including genotype, stage of microspore development, donor plant growing conditions, media composition, and culture conditions. One of the major factors involved in the orientation of the morphogenetic reactions of microspores grown on in vitro culture media toward embryogenesis, finalized with the obtaining of isogenic lines is the pH value of the culture media. pH, saw by literature as one of the stress factors that can cause a switch between the normal gametophytic path of immature pollen grain toward a sporophytic, embryogenic development, was investigated by many authors, the results being directly dependent on the species or genotype used in the experiments. Baillie *et al.* (1992) have shown that at *B. rapa* the highest number of embryos was obtained in media with a pH value of 6.2, while Glans *et al.* (1988) found that a pH of 5.7-6.0 was more suitable for the development of *B. napus microspores* (Hansen, 1994). In other species, such as tobacco, Touraev (1997) concluded that increasing the pH to a value of 7.0 could lead to a large number of haploid embryos, whereas in the case of cotton, the pH 7.3 is a promoter of embryogenesis microspores. According to the literature, the mechanism of action of pH is through its influence on the activity of invertase, translated by slowing or accelerating the cleavage of sucrose. In this way, any change in pH leads to different absorption of sucrose by microspores, allowing re-programming of microspores development toward embryogenesis and obtaining of double haploids plants.

Lichter (1982) first reported the successful application of microspore culture techniques to *B. napus*, thereby es-

Received 15 July 2013; accepted 28 August 2013

tablishing the basis of isolated microspore cultures for the *Brassica* genus. Subsequently, successful microspore culture in different cauliflower (*B. oleracea* 'Botrytis'), broccoli (*B. oleracea* 'Italica'), tronchuda cabbages (*B. oleracea* 'Costata'), kohlrabi (*B. oleracea* 'Gongylodes'), ornamental kale (*B. oleracea* 'Acephala'). Thomas *et al.* (2003) listed over 200 DH commercial cultivars from 12 crop species. Of those species, 49 cultivars were *B. napus* and two cultivars were *B. juncea*. This list is now outdated and incomplete as breeding organizations are retaining cultivar development information as proprietary (Dunwell, 2010). Although methods have been optimized to achieve the maximum response, it is clear that there exist significant genetic differences between genotypes (Mohammadi *et al.* 1999). Since the ability of microspores to undergo divisions and generate DH plants has a strong genotype dependency and there are relatively few report on *B. oleracea*, most of the studies being focused on *B. napus*, our objective was to accomplish a screening of microspore morphogenetic reaction under the influence of pH. The results obtained represent the foundation of a more in-depth study for the determination of a most efficient protocol for the cultivation of microspores.

Materials and methods

Plant growth conditions and biologic material

Four open pollinated genotypes (TRM 1, TRM 2, DL 20 and DM 56) provided by Vegetable Research and Development Station Bacau were 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 40C, 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.

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 °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 ° °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 1970) 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 °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.

During the present study 11 variants were tested to determine the optimum value of pH: V1 - 5,0; V2 - 5,2; V3 - 5,4; V4 - 5,6; V5 - 5,8; V6 - 6,0; V7 - 6,2; V8 - 6,4; V9 - 6,6; V10 - 6,8; V11 - 7,0. The control variant, as highlighted by literature was the pH level of 6.2, variant V7.

Cytological studies

For the determination of the medium influence over the orientation of developmental processes of *B. 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

Four open pollinated genotypes TRM 1, TRM 2, DL 20 and DM 56 were tested during two years to determine the influence of pH over the entire range of morphogenetic reaction of microspores, extremely important in determining the developmental pathway that the microspores will follow: viability, ability to generate callus, multicellular structure, embryo yield, percentage of normally formed embryos, etc. At all genotypes, the microspores viability was tested both at the time of inoculation and during the incubation time, after 10 and 20 days, using FDA method as described by literature. At inoculation stage on all tested variants the viability of microspores was near 90 - 92% most likely, the difference being given by microspores which suffered damage during the isolation, maceration and centrifugation processes.

After 10 days and 20 days, the percentage of viable microspores collapsed to variants of the culture medium in which the pH value was more than 6, leading to a total lack of viability on variants V9 - pH 6.6, V10 - pH 6.8 and V11 - pH 7.0. The death of microspores is due to the inhibition of the invertase activity under high level of pH and starvation of microspores as a result of the unavailability of carbohydrates source.

On the variants where the pH value was situated around 5.2 – 5.8 more than 50% of the microspores were viable Fig. 1, showing good signs of mitotic activity, with multicellular structures and embryoids. For DM 56 genotype, there was no significant difference in viability when pH increased from 5.8 to 6.4, 6.6 or 7.0, but no embryos were obtained when the pH was increased over 6.6. Our findings supports the results obtained at *B. napus*, where a pH of 5.8 - 6.1 was favourable for embryogenesis (Takahira, 2011), while at *B. campestris* the optimum pH was 6.6 (Baillie, 1992).

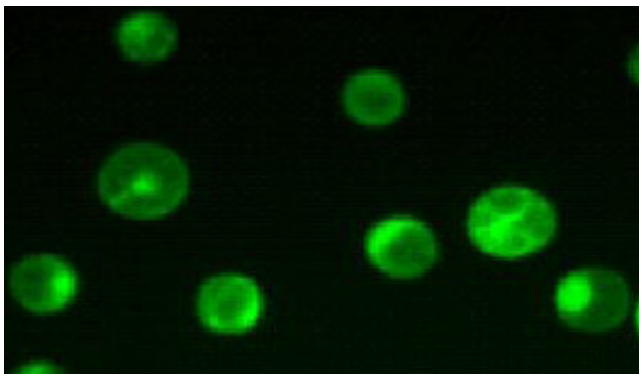

Fig. 1. Viable microspores on variant V5 (FDA staining - fluorescence microscope 100x)

The optimum pH value for sustainability of microspores viability, as well as for the switch of developmental processes toward embryogenesis is ranged between 5.4-5.8, variant V3-V5(Tab. 1). First cell divisions were observed inside microspore walls after three days of culture, the microspore population being asynchronous Fig. 2. The cytological analysis revealed initially the presence of two-three nucleus, their division becoming progressively more active, reaching up to 30 nuclei after the 10th day of culture.

Tab. 1. The viability of microspores after 20 days of culture on media with different values of pH

Nr. crt	Variant	pH	DL 20	DM 56	TRM 1	TRM 2
1	V1	5,0	++	++	+++	+
2	V2	5,2	++	++	++	++
3	V3	5,4	+++	++	+++	+++
4	V4	5,6	+++	+++	+++	+++
5	V5	5,8	+++	+++	+++	+++
6	V6	6,0	++	+++	++	+
7	V7	6,2	++	+++	+++	++
8	V8	6,4	++	++	++	+
9	V9	6,6	+	+++	+	-
10	V10	6,8	+	+++	+	-
11	V11	7,0	+	+++	+	-

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

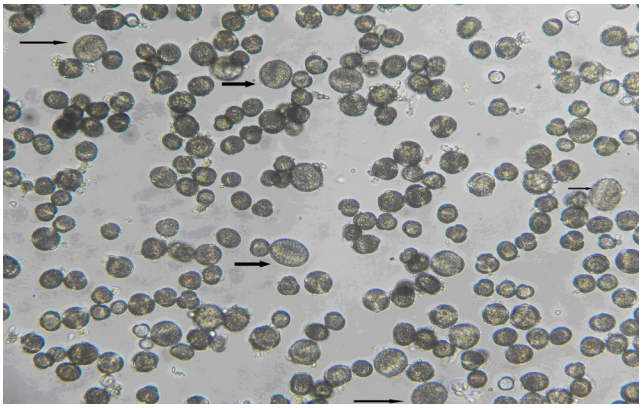

Fig. 2. Asynchronous population of microspores (40x) – arrows indicate embryogenic microspores

At 5.8 value of pH, multicellular colonies emerging from microspore walls were observed during the second week of culture, while no colonies were observed in microspores incubated on variants with pH higher than 6.2.

At lower levels of pH, the evolution of the microspores was oriented toward the apparition of star-like microspores and symmetric divisions but most of the multicellular structure development stopped by the third week of cul-

ture, and the yield of embryos were significant lower than on variants with a pH of 5.6-5.8.

Microspore-derived structures and embryoids were obtained in isolated microspore cultures of all four white cabbage genotypes. The analysis did not show genotypes to be statistically different, mean green embryo yields of the individual genotypes at different media pH levels showed varying results, in low limits Fig. 3. Only TRM2 showed increased embryogenic capacity at higher levels of pH, up to 6.6, while the others had remarkable response on lower of pH.

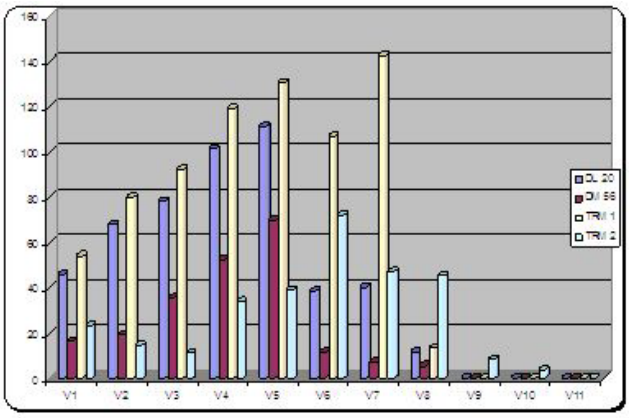

Fig. 3. Variation of normal embryo number yielded at different values of pH

When microspores were cultured in the above mentioned conditions, on NLN medium at 5.6-6.0 pH levels the microspores switch their developmental pathway toward the sporogenesis with the apparition of embryogenic microspores. These microspores larger than an average non-embryogenic microspore, with small vacuoles and the nucleus positioned in the central of cell, with cytoplasmic strands from the perinuclear to the subcortical cytoplasm divide symmetrically and gave rise to multicellular structures Fig. 4 after 4–5 days in culture.

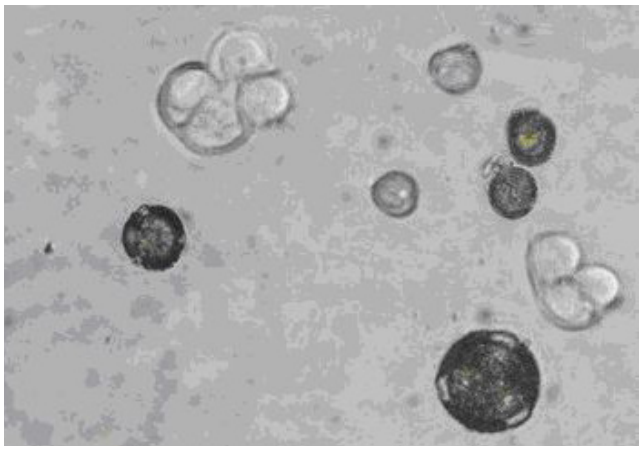

Fig. 4. Multicellular structures generated from *Brassica mcrospores* (100x)

The further developments of these structures are oriented toward either small callus cluster or multicellular embryos, visible with naked eyes within about three weeks of culture.

The development of the embryos is asynchronous, after 4 weeks of culture the population of embryos being formed from small globular embryos, heart-shaped embryos and well-developed cotyledonary embryos. The transfer of these embryos Fig. 5 on solid B5 medium Gamborg (1970) supplemented with PGRs - BAP- 8.9 μM and NAA 2.7 μM, 2 % sucrose and 0.08% agar-agar and held under light, allowed their further development by turning into green, initiating active root growth within 5 days, and subsequently developed into plantlets.

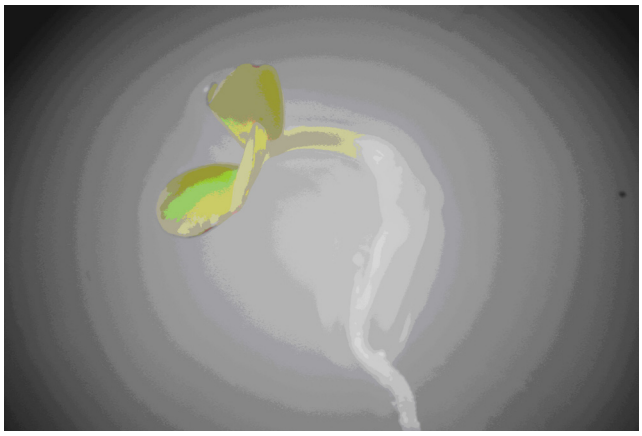

Fig. 5. Normal, mature embryo derived from microspore (40x)

The percentage of normally developed plants was more than 80% at all tested genotypes and after seven weeks the plantlets have been successfully transferred to soil.

Conclusions

The results of this study emphasize the importance of pH in the orientation of morphogenetic reaction of microspores toward embryogenesis and generation of double haploid plants. Somatic embryos were induced from microspore culture and successfully developed into healthy whole plants, nearly all of them being successfully acclimatized. Optimal value of pH was determined in four genotypes and represents an important achievement for the improvement of the protocol for an efficient and rapid production of completely homozygous plants of *B. oleracea.*

Acknowledgements

This work was co financed by the European Social Fund through Sectoral Operational Programme Human Resources Development 2007-2013, project number POSDRU/I.89/1.5/S62371 Postdoctoral Schole in Agriculture and Veterinary Medicine area".

References

Ahmadi B, Alizadeh K, Teixeira da Silva JA (2012). Enhanced regeneration of haploid plantlets from microspores of *Brassica napus* L. using bleomycin, PCIB, and phytohormones. Plant Cell, Tissue Organ Cult 109:525-533.

Baillie AMR, Epp DJ, Hutcheson D, Keller VA (1992). *In vitro* culture of isolated microspores and regeneration of plants in *Brassica campestris,* Plant Cell Rep 11:234-237.

Dunwell JM (2010). Haploids in flowering plants: origins and exploitation. Plant Biot J 8:377-424.

Hansen M (1994). Gametic embryogenesis in Brassica: optimization of production and germination of embryos. In: Javornik B, Bohanec B, Kreft I (eds) Proceedings of the international colloquium on impact of plant biotechnology on agriculture. Rogla, Slovenia, Centre for Plant Biotechnology and Breeding, University of Ljubjana, Slovenia, p. 15-18.

Lichter R (1982). Induction of haploid plants from isolated pollen of *Brassica napus*. E Pflanzenphysiol 105:427-434

Mohammadi PP, Moieni A, Ebrahimi A, Javidfar F (2012). Doubled haploid plants following colchicine treatment of microsporederived embryos of oilseed rape (*Brassica napus* L.). Plant Cell, Tissue Organ Cult 108:251-256.

Takahira J, Cousin A, Nelson MN, Cowling WA (2011). Improvement in efficiency of microspore culture to produce doubled haploid canola (*Brassica napus* L.) by flow cytometry. Plant Cell, Tissue Organ Cult 104:51-59.

Touraev A, Vicente O, Heberle-Bors E, (1997). Initiation of microspore embryogenesis by stress. Trends Plant Science 2: 285-303.