EFFECT OF CARBOHYDRATE SOURCE OVER THE ANDROGENESIS OF *BRASSICA OLERACEA* L. ANTHERS CULTIVATED *IN VITRO*

Tina Oana CRISTEA[1], Constantin LEONTE[2], Maria PRISECARU[1], Creola BREZEANU[1], Marian BREZEANU[1]

e-mail: tinaoana@yahoo.com

Abstract

Now-a-days plant tissue culture *in vitro* provides key opportunities for plant quality enhancement and subsequent economic sustainability. Haploid production of *Brassica* spp. through anther culture proved to be an important approach of tissue culture, during the last decades. Traditionally, plant breeders usually achieve homozygosity of the cross products by using the self-fertilization, a time consuming process. By anther culture, homozygous plant can be produced within a year as compared to the long inbreeding method, which might take 8-10 years. There are many factors that have been found to affect the ability of an anther to undergo successive changes in its developmental path in order to leave the gametophytic pathway and resort to a sporophytic mode of development. Among the factors that play a critical role in the orientation of morphogenetic reaction of anthers cultivated in vitro and ultimately in the regeneration of vitroplants at *Brassica* species the carbohydrate source and its concentration are considered to be of peculiar importance. The carbohydrates act as an energy source and as an osmotic regulator in the culture medium. Although sucrose or glucose represents the main sugars of choice in anther culture media, there are studies in which maltose turned out to be also a carbohydrate source suitable for androgenesis at different species. In the present study the four main types of carbohydrates (sucrose, glucose, fructose and maltose) and its concentrations were tested. The organogenic, embryogenic and calusogenic competences of *Brassica* anthers were highly influenced by the type of carbohydrate source added to NLN basic medium (Lichter, 1982) supplemented with BAP - 8.8 μM and 2.7 NAA μM. The results obtained in our study shows that sucrose proved to be the best for androgenic plant regeneration at *Brassica oleracea* with an optimal concentration of 0.09 M, followed by maltose and glucose, while fructose was less suitable for androgenesis sustainability.

Key words: sucrose, glucose, maltose, cabbage, haploids

Taking in consideration also the wild relatives, the genus *Brassica* is represented in vegetal regnum by almost 150 species. Among the cultivated species *B. napus*, *B. rapa* and *B. juncea* are utilized for oil production, while the varieties of *B. oleracea* are extensively cultivated as vegetables (cabbage, cauliflower, Brussel sprout, broccoli, kohlrabi).

Plant biotechnology and within its application, anther culture *in vitro* is an important tool for breeding activity oriented toward the enhancement of qualitative and quantitative traits of these important vegetable plants. Due to all the climacteric and nutritional constrains that we are facing now-a-days, the efforts of the breeders are focused on the obtaining of new high-yielded, resistant genotypes, more specific hybrids that by expressing their heterosis should better respond to market and climacteric request. Moreover, homozygous seeds and plants are essentially ideal materials for genetics, molecular biology, biotechnology, and also for plant breeding. They provide research and commercial genetic sources of which, within a variety, a largely identical genotype is guaranteed (Pechan, 2001). The obtaining of homozygous plants can be achieved through conventional selfing and backcrossing, which is a time-consuming process and production of haploids via ovule culture - gynogenesis, incongruous distant pollination such as the so-called "bulbosum" technique, pollination with irradiated pollen or pollination of irradiated pistils, anther and microspore culture – androgenesis (Cardoza, 2004). In general, microspore and pollen culture is one of the most frequently used methods to produce haploids in angiosperms. After spontaneous or induced doubling of chromosomes, fertile doubled haploid plants can be obtained.

The cultivation of plant cell and tissues *in vitro* requires the inclusion in the culture medium

[1] SCDA Bacău
[2] USAMV Iaşi

of a carbohydrate that is used as a source of energy, as a carbon substrate for different biosynthesis processes and as an osmoticum. The importance of sugars for *in vitro* cultured tissues is crucial due to the fact that their photosynthetic activity is significantly reduced during their life in these conditions. According to literature, sugars have important signaling functions through the entire life cycle influencing the plant development and gene expression. Due to all these characteristics the carbohydrates play a key role on the *in vitro* physiology, differentiation and growth of cells (Zimmerman, 1989).

Moreover, the type of carbohydrate included in the cultivation medium and its concentration determine different patterns of morphogenesis, thus influencing the plants ability to regenerate *in vitro* callus, shoots or embryo (Tang, 2003).

In *Brassica* crops, the studies regarding the influence of carbohydrate source over the ability of tissues to undergo different physiological and morphological changes that can lead to achievement of organogenetic and embryogenetic competences were mainly done on *Brassica napus* and *Brassica rapa*, regarded as model plant for these crops. Still the specie specific reaction must be addressed in order to be able to establish an efficient reproducible protocol for *in vitro* cultivation of *Brassica oleracea* anthers.

MATERIAL AND METHODS

Plant material

The experiments were performed in the Laboratory of Tissue Culture at Vegetable Research and Development Station Bacau during 2011-2012. The donor plants of *Brassica oleracea* L., breeding line TM51 were maintained in growth chambers at 16 h light and 8 h dark photoperiods, at 18±0.5^{0}C and 60% relative humidity, with a proper regime of watering, fertilization and pest control. The flower buds were collected from racemes where one or two flowers had reached anthesis. Only the buds of 3.0 – 3.4 mm in length were utilized in experiments as at this size the anther's microspores are at the late uninucleate stage of development (as previously determined using 1% aceto-carmine under microscope).

Sterilization

The sterilization of explants was accomplished by washing thoroughly under running tap water for 30 min and treatment with a surfactant, Tween 20 (10 drops per 100ml of sterilized distilled water). Later these explants were surface sterilized with 0.1% mercuric chloride (w/v) for 15 min and repeatedly washed using sterilized distilled water.

Culture medium

For the determination of carbohydrate influence over the organogenic, embryogenic and calusogenic competences of *Brassica* anthers, 12 variants in three replicates were utilized. The basic culture medium utilized in our experimentations was NLN medium (Lichter, 1982) supplemented with BAP - 8.8 µM and 2.7 µM NAA. The pH was adjusted to 5.8, by adding few drops of NaOH 1N, prior to the addition of 8.0 g/l agar and autoclaved at 121°C (1.06 kg/cm^2) for 25 min.

Culture technique

Under aseptic conditions, anthers were removed from the sterilized buds using a fine Tweezers *(fig. 1)*. The anthers were inoculated on sterile tubes with culture media containing different mineral composition formula – as illustrated in *table 1*.

Table 1

Experimental variants utilised for the determination of carbohydrate effect over the organogenic, embryogenic and calusogenic competences of *Brassica* anthers

Variant	Sucrose	Glucose	Fructose	Maltose
V1	0.06 M	-	-	-
V2	0.09 M	-	-	-
V3	0.12 M	-	-	-
V4	-	0.11 M	-	-
V5	-	0.17 M	-	-
V6	-	0.22 M	-	-
V7	-	-	0.11 M	-
V8	-	-	0.17 M	-
V9	-	-	0.22 M	-
V10	-	-	-	0.06 M
V11	-	-	-	0.08 M
V12	-	-	-	0.36 M

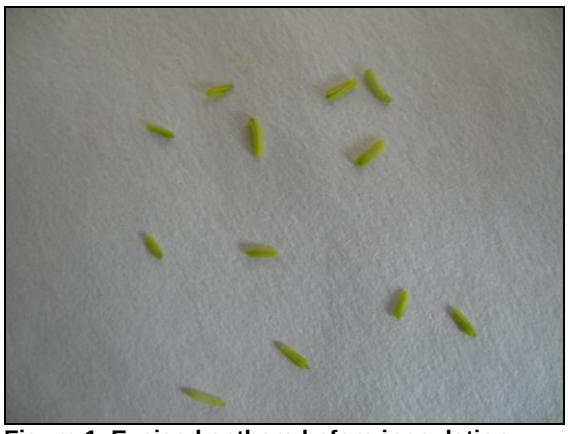

Figure 1 Excised anthers before inoculation

Figure 2 White-greenish callus with regenerative competence

The cultures were incubated at 33°C temperature for one week in complete dark, and then transferred in culture chambers with controlled light, humidity and temperature control at 25^0C, a 16-h photoperiod, and 5000 lx light intensity. The transfer on new fresh media was accomplished every four weeks plate inside the laminar airflow cabinet. The culture vessels showing signs of contamination were discarded. Day to day observation was carried out to note the responses.

Adventitious shoots obtained on each medium were separated from explants and cultured individually on new fresh media for the continuation of regeneration processes. Normal and healthy regenerated shoots (2.5–3 cm in length) with at least two expanded leaves were excised from explants and transferred to culture tubes containing NLN medium (Lichter, 1982) supplemented with 2.7 µM NAA. The cultures were incubated in a growth chamber under the same conditions as described above. The rooted plants were acclimatized and planted in a potting mixture of sterilized sand + vermiculite (1:1 ratio) in plastic cups, hardened in a mist chamber (80% relative humidity) for 2 weeks before transfer to green house.

During the development of experiments we emphasize over the number of anthers producing callus, embryoids and organogenic meristematic centers. The frequency of direct and indirect organogenesis and embryogenesis reaction and the frequency of reacted anthers were calculated in percentage to the *in vitro* initial explants.

RESULTS AND DISCUSSIONS

The results obtained confirm that the source of carbohydrate is one of the major supplements that support the development of anthers finalized either to callus formation, or shoot and embryo regeneration. Both the type of carbohydrate and the concentration gave high variation of tested parameters, observation that strengthen the idea that sugars have important signaling functions influencing the plant development and gene expression.

In order to determine the best source of carbohydrates we utilized in our experiments four main types of carbohydrates: sucrose, maltose, glucose and fructose in different concentrations. The first visible changes in cultured anthers were a slight enlargement in size within the first week after inoculation. The main type of morphogenetic reaction was oriented toward the formation of callus, and here we had variations regarding its texture, color and subsequently its competence to generate embryo and shoots. The formation of callus started mainly on the filament side of the anthers its further evolution being dependent on the experimental variant tested.

Thus, on determine two forms of callus: one, with a more compact structure, white-greenish coloured, with isodiametric cells which allowed the regeneration of shoots and embryo *(fig. 2)* and a more laxed one, with a high rate of growth, but with no regeneration competence.

Regarding the frequency of callus formation on the twelve variants tested in the present study, the results shows that sucrose promotes the proliferation of callus during the entire period of cultivation, supporting the initiation and development of regenerative structures (*fig. 3)*, glucose at initial stages, followed by a stagnation of growth, without any beneficial effect over regeneration, while fructose is less appropriate for anther cultivation. Maltose is also a promoter of callus growth and regeneration, but still with lower

results than sucrose. The most efficient concentration of sucrose was 0.09M, the increase in quantity reflected in a decrease of callus formation percentage. The effect of sucrose on the callus induction frequency might be due to its contribution to the osmotic potential of the medium rather than its utilization as a carbon source.

Figure 3 The development of regenerative structures from callus tissue

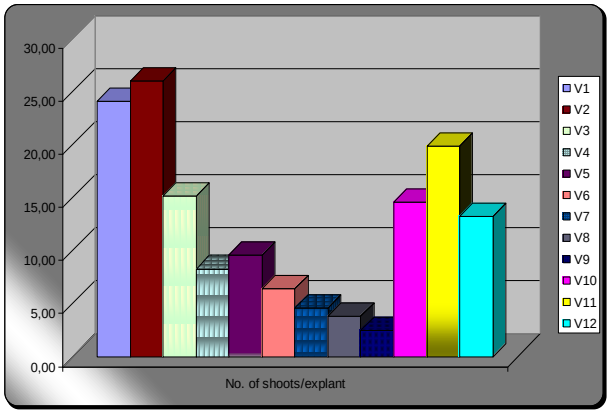

Figure 4 Graphical representation of evolution of shoot regeneration in correlation with type and concentration of carbohydrate source utilised in culture medium

Linking the type of carbohydrates and its concentrations on concluded that the highest number of shoots per explant - 25.96±0.34 was obtained with 0.09M sucrose. With 0.06 M sucrose, shoot proliferation was slightly lower - 24.11±0.41, while an increase of concentration to 0.12M lead to a decrease of shoot regeneration frequency to 15.13±0.34. Adding glucose or fructose instead of sucrose resulted in a reduction in the regeneration efficiency of anthers. The lowest results were obtained on variant V9, characterized through a concentration of 0.22M fructose, where only 2.50±0.30 shoots per explant were obtained, comparing with 6.42±0.75 shoots/explant obtained when the concentration of fructose was dropped down to 0.11M. One explanation of this decrease can be the inhibitory effect of high carbohydrate concentrations that may lead to less water potential of the medium, which inhibited the cell growth and development.

The same results were obtained when maltose was used as carbohydrate source in culture medium. The number of shoots increased with concentration (*fig. 4)*, until a certain level and on variant with 0.36M the shoot regeneration decreased.

CONCLUSIONS

Both the type of carbohydrate and the concentration highly influence the callusogenic, organogenic and embryogenic competence of *Brassica oleracea* L. anthers cultivated *in vitro.*

Among the four types of carbohydrates tested in the present study, sucrose promotes the proliferation of callus and shoots development during the entire period of cultivation, supporting the initiation and development of regenerative structures. The most efficient concentration of carbohydrate proved to be 0.09M, higher concentration leading to a decrease in the regenerative competence of anthers.

ACKNOWLEDGMENTS

This work was cofinanced from 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

Pechan PM, Smykal P, 2001 - *Androgenesis: Affecting the fate of the male gametophyte.* Physiol. Plant, 111, p. 1-8.

Cardoza V, Stewart CNJR, 2004 - *Brassica biotechnology: Progress in cellular and molecular biology.* In Vitro Cell. Dev. Biol. Plant. 40: 542-551.

Spomer, L. A.; Smith, M. A. L. 1996 - *Direct measurement of water availability in gelled plant tissue culture media.* In Vitro Cell. Dev. Biol.–Plant 32, p. 210–215.

Tang, G.X., W.J. Zhou, H.Z. Li., B.Z. Mao and Z.H. He. 2003 - *Medium, explant and genotype factors influencing shoot regeneration in Oilseed Brassica spp. Mao*, J. Agronomy & Crop Science, 189, p. 351-358.

Zimmerman, T. W.; Cobb, B. G., 1989 - *Vitrification and soluble carbohydrate levels in Petunia leaves as influenced by media, gelrite and sucrose concentration.* Plant Cell Rep. 8:358–360.