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IN VITRO CULTURE OF UNFERTILIZED OVULES IN *BRASSICA OLERACEA* L.

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Key words: *Brassica oleracea* var. *capitata*, *ovule culture, embryogenesis, haploid plants.*

INTRODUCTION

Brassica vegetables are heterozygous cross-pollinated crops. Their haploidy provides a rapid method for producing pure lines that can be used as parents in creating hybrid cultivars. Anther culture has now been widely adopted by a number of seed production firms in the world, involved in *Brassica* crop breeding (6,7). The broccoli hybrids produced from crosses of DH plants were superior in many traits to commercial hybrids, which were developed through conventional inbreeding methods (7).

The use of haploids in breeding has as purpose, on one hand the maintenance and selection of top genomes and on the other hand the discovery of the best forms that that present heterozygosis interaction (1,2,3,4,5). In the present paper we are presenting an efficient method for the obtaining of cabbage plants (*Brassica oleracea* var. *capitata*) through the culture of unfertilised ovules, an alternative method of obtaining the haploids, less mentioned in reference materials of *Brassica*, but which determined the successful obtaining of plants and isogenic lines in many species (7).

MATERIAL AND METHODS

The biological material used in our research is represented through immature inflorescences of cabbage harvested approximately 3-4 days before flowering that belongs to the genotypes Royal-Delphy F1 (simple hybrid parental males used to obtain double hybrid Flavius) BCO 7-10 (advanced inbred line, homozygous C6 generation), Z2 and Z2-12-2 (advanced inbred lines, homozygous generation C5). The assessment of inoculation stage of the embryo sac (mature embryo sac) was realised based on indirect criteria: floral button size of 2.5-3 mm; the size and shape of the egg (the pear shaped ovule, occupies 2/3 of the ovarian cavity) size and colour of anthers (0.75 to 1 mm, green) pollen development stage (uninucleate, late microspore phase).

Flower buttons sterilization was carried out by immersion in mercury chloride ($HgCl_2$) 0.1%, for 10 minutes, followed by several washes with sterile distilled water. After washing, the eggs were removed by dissection of ovarian wall under binocular loupe, followed by their placement on induction media. To induce gynogenesis 8 culture media hormone variants (V1-V8) were used, all of them derived from basic Murashige-Skoog medium (MS, 1962) in which the vitamin complex MS was replaced with B5 (Gamborg, 1968) (tab. 1).

The ovule cultures were incubated under a 16 hours photoperiod (at 3000 lx) at a temperature of 25^0C. The embryoid and callus structures were transferred to differentiation mediums noted as R1-R8 (table 2), under the same culture conditions (table 2). The gynogenetic products, embryos and plants were then placed in the same culture medium (MS with vitamin B5) without phytohormones, and with 2% sucrose. The acclimatization of plants obtained from septic conditions was accomplished gradually by passing through a hydroponic system and then in pots with sterilized soil (soil / perlite 1/1). After accommodating, the potted plants were transferred to the greenhouse and then in the experimental field.

The histological study was performed by the classical method of serial sections (Navaşin-Brown fixation, included in paraffin, sectioned at 7μ, hydrated and coloured with hematoxylin Erlich). Chromosome number and structure were determined on metaphases of regenerated plant root meristems. The processing of biological material was realised according to classical Feulgen method.

RESULTS AND DISCUSSIONS

After approximately two weeks of culture, the ovules began to form callus on variants 1 and 4, and than on all other variants, with different intensities (table 1). The callus appearance was different, depending on the culture medium variant. Thus on variants V1, V2, V5, V6 and V7, the obtained callus was friable, yellow-green (Fig. 1) while hard consistency callus yellowish-white was obtained on the variants V3, V4 and V8. After another two weeks, the friable callus, yellow-green coloured began to form meristematic centres and small embryos on variants R1, R2, R5 and R6 (figures 2,3,5). The response of the 4 genotype on the regenerative media was recorded in table 3. Passed

on the culture medium MS (1962) without phytohormones, under 16 hours photoperiod (2500 lx), the embryonic structures obtained on R1 and R2 variants have evolved into complete plants with roots (figures 4,5).

Not all the genotypes showed the same intensity in the production of plants with gynogenetic origin. The callus of genotypes Royal-Delphy and Z2-12 showed a high capacity for proliferation and formation of embryonic structures (Figures 3,5), especially on R1 and R2 mediums. Each callus generated from one ovule 20-25 embryos, which mostly evolved in plants. Genotypes BCO7-10 and Z2-2 showed a lower capacity both to form a callus and to generate embryos and plants (table 4).

In the embryogenic cultures the formation of abnormal embryos was observed: fused embryos, cotyledons or embryos united with one cotyledon hypertrophied. Most of them have degenerated or evolved in abnormal plants that did not survive. However, a large number of plants were regenerated and acclimatized to septic conditions, first in hydroponic culture conditions, and than in pots, greenhouse and field. Percentage of adaptability was satisfactory 68%, respectively. Also, during growth in most plants with gynogenetic origin, there was noticed differences between them and seed born plants, materialized by a lower vitality, smaller size and a pale green color of the leaves.

The cytogenetic study realized in root meristems of plants regenerated from unfertilized eggs showed a predominant number of haploid plants, as well as diploid and mixoploid plants (table 5). The presence of diploid and mixoploid plants demonstrates the instability of haploid genome and the spontaneous duplication of chromosomes.

In addition to experimental androgenesis, the gynogenesis by unfertilized ovule culture appears to be an important method for the efficient production of haploid plants in *Brassica oleracea* var. *capitata*. In case of the genotypes studied in the present study, the best combination of plant hormones to induce embryogenic callus was 0.3 mg / l BAP, 0.1 mg / l NAA and 0.2 mg / l 2,4-D (V6) while for the induction of embryogenesis the best were variants with1-2 mg / l BAP (R1 and R2). For the development of embryos in plants the phytohormones are not required. There was no genotype with the ability to form embryos directly from ovules (direct embryogenesis).

Table 1. The nutritive medium used for in vitro culture of unfertilised ovules of *Brassica oleracea* L.

Components	**V1**	**V2**	**V3**	**V4**	**V5**	**V6**	**V7**	**V8**
Macroelements	MS	MS	MS	MS	MS	MS	MS	MS
Microelements	MS	MS	MS	MS	MS	MS	MS	MS
Vitamins	B5	B5	B5	B5	B5	B5	B5	B5
BAP	-	0,1mg/l	-	1mg/l	-	0,3mg/l	0,2mg/l	-
Kinetin	-	-	-	-	1mg/l	-	-	0,1mg/l
NAA	0,1mg/l	-	1mg/l	-	-	0,1mg/l	0,1mg/l	-
IAA	-	-	-	0,1mg/l	-	-	-	-
2,4 D	0,1mg/l	0,5mg/l	1mg/l	0,5mg/l	1mg/l	0,2mg/l	-	0,5mg/l
Saccharose	100g/l	30g/l	30g/l	30g/l	30g/l	30g/l	30g/l	30g/l
Serine	-	100mg/l	50mg/l	-	-	-	-	-
Glutamin	500mg/l	-	-	-	-	-	-	-
Agar-agar	8g/l	8g/l	8g/l	8g/l	8g/l	8g/l	8g/l	8g/l
pH	5,8	5,8	5,8	5,8	5,8	5,8	5,8	5,8

Table 2. Variants of the regeneration culture medium

Components	**R1**	**R2**	**R3**	**R4**	**R5**	**R6**	**R7**	**R8**
Macroelements	MS	MS	MS	MS	MS	MS	MS	MS
Microelement	MS	MS	MS	MS	MS	MS	MS	MS
Vitamins	B5	B5	B5	B5	B5	B5	B5	B5
BAP	1mg/l	2mg/l	-	-	2mg/l	1mg/l	-	-
Kinetin	-	-	1mg/l	2mg/l	-	-	1mg/l	2mg/l
NAA	-	-	-	-	0,5mg/l	-	0,5mg/l	-
GA_3	-	-	-	-	-	0,01mg/l	-	0,01mg/l
Saccharose	30g/l	30g/l	30g/l	30g/l	30g/l	30g/l	30g/l	30g/l
Agar-agar	8g/l	8g/l	8g/l	8g/l	8g/l	8g/l	8g/l	8g/l
pH	5,6	5,6	5,6	5,6	5,6	5,6	5,6	5,6

Table 3. Gynogenetic callus reactivity on variants of the regeneration culture medium

Nr. crt.	Genotip	R1	R2	R3	R4	R5	R6	R7	R8
1	Royal-Delphy	embryos	embryos	-	-	embryos	embryos	-	-
2	BCO7-10	embryos	embryos	-	-	-	-	-	-
3	Z2-12	embryos	embryos	-	-	embryos	embryos	-	-
4	Z2-2	embryos	embryos	-	-	-	-	-	-

Table 4. Induction rate of embryos and plants from gynogenetic callus

Nr crt	Genotype	No. inoculated ovule	No. obtained calluses	No. calluses with embryoids	Average no. of embryos/callus	No. regenerated plants	%
1	Royal-Delphy	200	98	80	20	134	67
2	BCO 7-10	200	88	56	10	118	59
3	Z2-12	200	108	92	25	163	81
4	Z2-2	200	76	57	8	90	45

Table 5. The ploidy level of the plant regenerated from ovules of Brassica oleracea L.

Genotype	No. of analysed plants	n	2n	mixoploids
Royal-Delphy	5	3	1	1
BCO7-10	5	3	-	2
Z2-12	10	7	2	1
Z2-2	5	4	1	-

ABSTRACT

Using the unfertilised ovule culture of 4 genotypes of *Brassica oleracea* var. *capitata* (three consanguine lines and one hybrid) haploid plants diploid and mixoploid plants with gynogenetic origin were successfully obtained. A larger number of embryos/callus/ovules and normal plants were obtained. The gynogenesis in vitro through unfertilised ovule culture can be an effective method of production of haploids and dihaploids at white cabbage.

REFERENCES

1. PRISECARU M., CRISTEA T.O. 2007 - Comparative study to induction of haploid plants by "in vitro" anther and ovule culture of tomato (Lycopersicum esculentum L.). Studii şi Cercet. Ştiinţ. Biologie (Scientific Studies and Researches. Biology) 12: 41-43;
2. PRISECARU M., CRISTEA T.O., 2011 - Observations regarding the outturn of experimental gynogenesis at wheat (Triticum aestivum L.) and sugarbeet (Beta vulgais L.) Studii şi Cercetări Ştiinţifice. Biologie. (Scientific Studies and Researches. Biology) 20: 74-76;
3. PRISECARU M., CRISTEA T.O., 2009 - Induction of haploid plants from the female gametophyte of Triticum aestivum L., Studii si Cercetari Stiintifice. Biologie. (Scientific Studies and Researches. Biology) 16: 63-69;
4. PRISECARU M., CRISTEA T.O., 2009 - In vitro gynogenesis in Solanum tuberosum L., Studii si Cercetari Stiintifice. Biologie. (Scientific Studies and Researches. Biology) 16: 26 – 31;
5. PRISECARU M., GIORGHITA G., 1995 - Establishment of regeneration capacity by „in vitro" cultures in Brassica oleracea L. Analele Şt. ale Univ. „Al. I. Cuza", Iasi, tomul XLI, 2: 85-88;
6. PRISECARU M., GIORGHITA G., MIHU G., NICUTA D, CRISTEA T. O., 2000 - Production of haploid plants by in vitro anther and ovule culture of Brassica. Ed.Corson, Genetics and Evolutions, 4:169-1;
7. PRISECARU M., CRISTEA T.O., BADALUTA N., 2013 - Plant regeneration from anthers culture of Brassica oleracea L. Studii si Cercetari Stiintifice. Biologie. (Scientific Studies and Researches. Biology) 22/1: 43–46.

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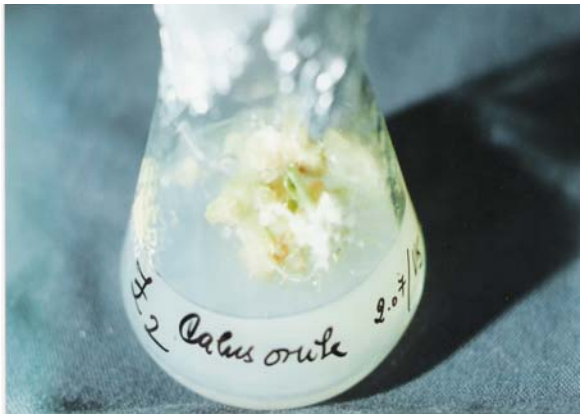

Fig. 1. Callus obtained from ovules

Fig. 2. Differentiation of embryonic structures

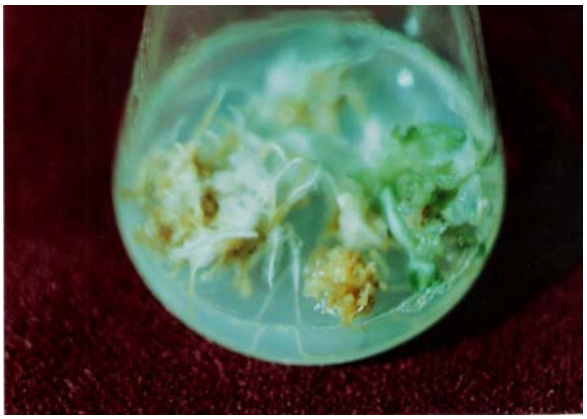

Fig. 3. Development of embryos in plants

Fig. 4. Plants with gynogenetic origin

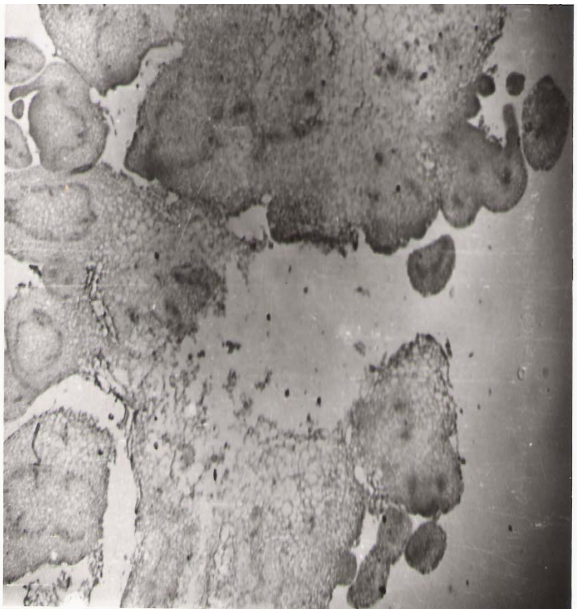

Fig. 5. Embryonic structures developed in the gynogenetic callus

Fig. 6. Complete plant developed from gynogenetic callus