MORPHOGENETICAL AND HISTOLOGICAL STUDIES OF „IN VITRO” ANTHER CULTURES OF *BRASSICA OLERACEA* L.

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Abstract. The anthers were inoculated on 8 hormonic variants of MS (Murashige-Skoog, 1962) medium and on one variant of B5 (Gamborg, 1968) slightly changed medium formula. The main morphogenetic reaction was callus formation. The callus's consistency and colour differed depending on the hormonic variant. The friable callus for all the tested genotypes had caulogenetic capacity. Its passage on differentiation media comprising cytokinins led to new shoots formation, the intensity of this phenomenon varied with the genotype and with the initial medium that provided the respective callus.

The histological studies of calluses or of anther shoots pointed out callus structure and the numerous histogenetic stages due to growth regulators, as well as in vitro regenerated shoots' structure.

INTRODUCTION

Brassica oleracea L is a vegetable species belonging to the *Cruciferae* family. The ancestor of today cabbage is *B. oleracea* L. ssp. *silvestris*, a species that provided a great number of varieties and forms over the centuries.

Brassica oleracea L. is appreciated from the economical viewpoint due to its high nourishing value occupying an important role in human diet and also for its therapeutic effects.

It is well – known that cabbage juice's content rich in sulphur, chlorine, calcium, iodine, iron and vitamins is indicated to cure duodenal ulcer, constipation, skin rashes, also in the diet of patients suffering from ostheoporosis and anemia, and its combination with carrote juice offers an excellent vitamin and mineral salts source.

Of all the numerous varieties of *Brassica oleracea* L. we mention the most known and used world wide: white cabbage (variety *capitata*, form alba Lam.), broccoli (variety *botrytis* L., subvariety *cymosa* Lam.) and cauliflower (variety *botrytis* L., subvariety *cauliflora* Alef.).

During our investigations we observed the morphogenetic reaction of the in vitro cultured anthers belonging to several genotypes of the a small number of plants were provided (with different levels of ploidy) by anther culture of several species of *Brassica* (Keller şi colab., 1984, Dias, J., C., 1999), but the most efficient method was microspore culture (Adamus, A., Samek, L., 2003). This method favoured the regeneration by microspore culture of *B. rapa*, *B. campestris* (Keller et al., 1975; Keller and Armstrong, 1979), *B. chinensis* (Chung et al., 1977) and *B. pekinensis* (Teng and Kuo, 1977).

MATERIAL AND METHODS

As biological material we used in our research floral buds from the inflorescences harvested from the Vegetable Research Centre of Bacau. We tested 12 genotypes of white cabbage (Z2; Z2-12; RM1; G37; 2TC – 19; TRM1; TRM2; DE; BR-4; BCO-7-6; BCO-7-10, BCO-076), 4 genotypes of broccoli (BR-312-3; BR-312-5; BR-11-2; BR-S) and one of cauliflower (CT-Bc).

The sterilisation of biological material was proceeded by emerging it into mercury chloride (solution 0.1 ‰) followed by repeated rinses with sterile distilled water.

Subsequently the anthers were excised from the floral buds and inoculated on many hormonic variants of the culture media. 8 of them comprised the basal MS medium (Murashige-Skoog, 1962) and only one contained the basal B5m (Gamborg, 1968) modified by Lillo and Shamin, 1983 (table 1)

Table 1. Hormonic formuli to prepare the initiation media

Nr.	Hormonic formuli	Basal medium	Growth regulators (mg/l)					
			IAA	IBA	NAA	2,4-D	BAP	KIN
1.	BB_2	MS	-	0,1	-	-	1	-
2.	BD	MS	-	-	-	0,5	1	-
3.	ND	MS	-	-	0,1	0,1	-	-
4.	BAD	MS	0,1	-	-	0,5	1	-
5.	KD	MS	-	-	-	1	-	1
6.	BDN	MS	-	-	0,1	0,02	0,3	-
7.	BN	MS	-	-	0,1	-	0,5	-
8.	BA	MS	0,1	-	-	-	1	-
9.	ND	B_5	-	-	0,1	0,1	-	-

After inoculation, the anthers were kept in darkness for 6 days at 35°C, then placed in an in vitro culture room (photoperiod of 16 hours), at a temperature of 23°C.

The general reaction of the initiation media was callus formation, which was then transferred on media to induce caulogenesis (table 2).

Table 2. Nourishing media used for differentiation

Nr.	Basal medium	BAP mg/l	KIN mg/l	GA3 mg/l
1.	MS	0,5	-	-
2.	MS	1	-	-
3.	MS	2	-	-
4.	MS	0,5	0,5	-
5.	MS	1	-	0,1

The shoots obtained were passed on hormone-free MS to produce roots.

Neoplantlet accommodation to septic media was accomplished in a hydroponic system. Afterwards they were transferred in pots containing a mixture of soil and perlite.

For histological analysis of calluses or anther shoots of the three varieties of B. oleracea species we used microscopic preparations of vegetal material included in paraffin. Microscope analysis was meant to establish callus structure and the varied histogenetic aspects appeared under the influence growth regulators and also the in vitro provided shoot structure.

RESULTS AND DISCUSSIONS

After being kept in darkness and at a high temperature for six days, most part of the anthers provided a small-sized, quite friable, white callus. The anthers were passed to light and two weeks after their inoculation on the initial media the anther callus had a different evolution depending on the the hormonic variants and especially on the genotype. The three varieties of Brassica formed two types of callus (of different consistency): friable and compact, that belonged to 2 categories: green and cream coloured.

The morphogenetic reaction of anthers from the 12 genotypes of white cabbage inoculated on induction media varied with the genotype and also with the hormonic content of the nourishing medium. Most frequently a friable, green or light green callus appeared and it had the highest regenerative capacity, providing shoots either directly on the induction medium or at the moment of its transfer on a medium formula comprising only cytokinins (fig. 1). This type of callus proliferated highly, particularly on the initial variants: BDN and BN, and those passed on BD (BAP – 0,5 mg/l, 2,4-D – 0,5 mg/l) and BAD (BAP – 1 mg/l, IAA – 0,1 mg/l, 2,4 – D – 0,5 mg/l) had a medium proliferation speed. Regarding the genotypes, the anthers of genotypes RM1 and Z2-12 were the most efficient to provide and proliferate the caulogenetic callus and the anthers of BCO-076 displayed a low capacity to produce this androgenetic structure (graphic nr. 1).

Generally, placing the friable green callus on the differentiation media (regardless the genotype) provided shoot regeneration (fig 2). This phenomenon’s intensity varied, the highest shooting percentage was obtained on B2 variant (2 mg/l BAP), followed by BK (1 mg/l BAP and 1 mg/l kinetine).

Compact green callus of hard or semi-hard consistency, was provided mostly by the anthers inoculated on BD, BA, KD and BAD, but the proliferation speed was not high. The genotypes DE, Z2-12, BCO-7-6 and 2TC19 manifested a good caulogenetic reaction on the above-mentioned medium variants. The meristematic centres were rendered evident on anther callus produced on BA and KD, but the transfer of this type of callus on differentiation media did not lead to shoot regeneration; the callus maintained its consistency. At contact surface between medium (rich in cytokinins) and callus, the latter generated long roots endowed with secondary branches, covered by absorbent hairs.

Similarly to the case of friable green callus, a quite great number of anthers provided a friable cream, whitish-cream or cream-yellowish callus. Although on some hormonic variants this type of callus proliferated intensely and it manifested a low caulogenetic capacity (though it was transferred on the same medium formuli to differentiate). Root formation was induced on BA, ND, KD and BAD on the same type of callus, with short roots grouped in tufts.

A small number of inoculated anthers generated a hard cream callus, characterised by an average and low proliferation speed, with an ability to produce just roots. It was noticed on the anthers placed on the following medium variants: ND (genotypes: DE, TRM2), BAD (genotypes: BCO-076, BCO-7-10, Z2), KD (genotypes: RM1, BR-4, G37) and BA (genotypes: Z2-12, Z2). Transfering this type of callus on media comprising only citokinins resulted in its low proliferation, sometimes accompanied by roots with an intense growth on the medium surface and also inside the culture medium.

Of all 12 genotypes of white cabbage, a small number of anthers from the genotypes Z2-12 and TRM1 provided plantlets directly on the initiation media, on KD, BN and ND (B5m) , (fig. 3).

Anther reaction for broccoli (4 genotypes) was callus formation (the same types): friable, green or cream coloured, and compact, also green and cream. Regarding the callusogenetic capacity, the anthers from the genotypes BR-312-3 and BR-312-5 proved to be the most efficient.

The friable green callus was provided only on two hormonic variants of the initiation media: BN and BDN (fig 4). This callus was characterised by high proliferation speed for the majority of genotypes. Most of the calluses had meristematic centres, the most numerous on BDN (graphic nr. 2).

Shoot regeneration from these androgenetic structures was evident on the differentiation medium B2.

The compact, green callus appeared less frequently than in the case of white cabbage. The anthers of genotypes BR-312-3 and BR-312-5 of broccoli placed on medium variant BAD generated a compact, small-sized, green callus. The same kind of callus, but with a higher proliferation, was provided by the anthers of BR-11-2 on BDN medium, on this callus being also rendered meristematic centres, but caulogenesis couldn’t be induced, not even when the calluses were transferred on differentiation media.

The friable, cream callus was also characterised by an intense proliferation, being produced by a great number of anthers belonging to BR-312-3 and BR-312-5. It mainly displayed rhyzogenetic capacity, a process that was visibly increased after the transfer on media comprising only cytokinin-like hormones. The cytokinins from the differentiation media also favoured the regeneration of a small number of shoots derived from the friable cream callus.

As for the subvariety cauliflora, the hormonic combination of the 9 medium formuli provided the same two types of callus on anthers. In the case of cauliflower, the best proliferation speed and caulogenetic capacity was also rendered evident for the friable green callus (fig. 5). The

hormonic variants for this callus's induction in a high percentage were: BDN and BN (graphic nr.3).

Anther inoculation on media comprising only auxins (ND with 0,1 mg/l NAA and 0,1 mg/l 2,4-D) favoured a foamy, high-proliferative, cream callus that proved to be rhyzogenetic. In the case of the same hormone combination (ND), but with a basal medium modified – B5, the reaction was formation of friable, low-proliferative, cream callus with roots and shoots (some shoots having inflorescences).

The transfer of friable cream callus on organogenetic media proved its caulogenetic capacity, only the callus formed on ND formula (B5m) generated a greater shoot number. We also noticed that in the case of friable green callus provided by cauliflower anthers the presence of a high amount of cytokinin (2 mg/l) in the differentiation media favoured the regeneration of a greater shoot number.

The aspect of the in vitro regenerated shoots was normal, each stem having 4 to 6 leaves with an alternate disposition. A small percentage was represented by individuals with chlorophyllian deffiency, albine or even shoots with leaf malformations (concrescent, very large and crisp leaves), but they did not survive (fig.6, 7).

Shoot accommodation to ex vitro medium was accomplished in a hydroponic system and it took about 2 weeks, the survival rate was of 80%. After accommodation the neoplantlets were transferred to soil pots (fig. 8).

Histoanatomical studies pointed out the presence of histogenetic structures that provide vascular elements and also of some organogenetic structures (either caulogenetic or rhyzogenetic) within androgenetic calluses (fig. 9, 10, 11, 12, 13, 14).

The shoots generated by anther callus displayed an almost normal structure.

CONCLUSIONS

The main morphogenetic reaction was callus formation, the newly formed callus having a consistency and colour that differed with the genotype and with the hormonic balance in the nourishing media.

Of all the four categories of callus that appeared, the friable green callus had the displayed the highest regenerative capacity.

The best proliferation speed of this type of callus was noticed on BDN, BN and BD media.

The friable cream callus (that had the lower regenerative capacity), proliferated intensely on BAD, BA and KD variants.

The most intense callusogenetic reaction was observed at genotypes RM1, DE, Z2-12, BCO-7-10, BCO-076 (for *capitata* variety) and BR-312-3, BR-312-5 (subvariety *cymosa*)

The phenomenon of shoot regeneration from androgenetic calluses was best represented on differentiation medium comprising 2 mg/l BAP

Genotypes TRM1 and Z2-12 provided plants from anthers on the following media: KD, BN and ND (B5m)

Histo-anatomical studies made on anther callus evinced the presence of some histogenetic and organogenetic structures.

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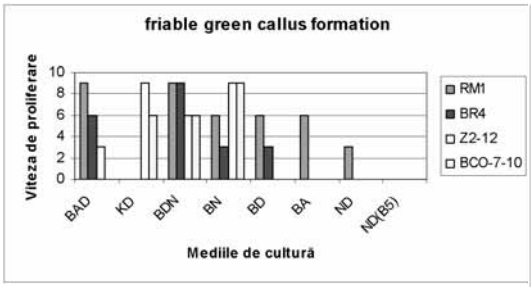
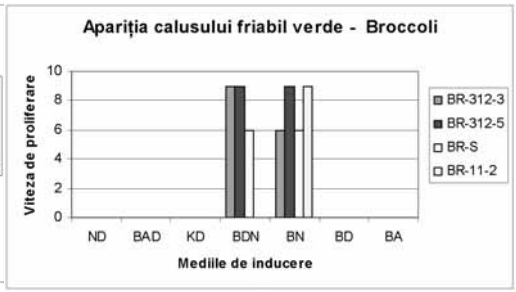
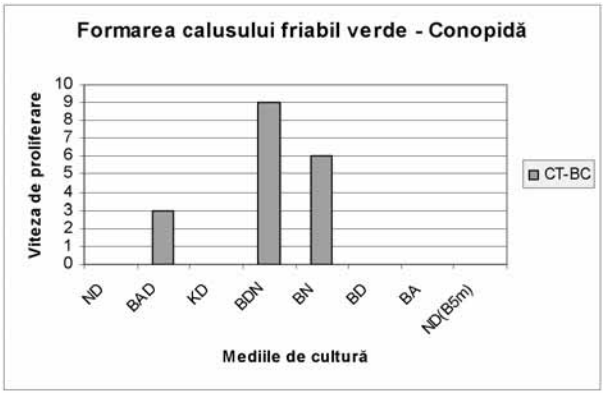


Graphic 1 – Generation and proliferation of friable green callus from white cabbage anthers



Graphic 2 – Proliferation of friable green callus from the anthers of broccoli



Graphic 3– Friable green callus formation from cauliflower anthers

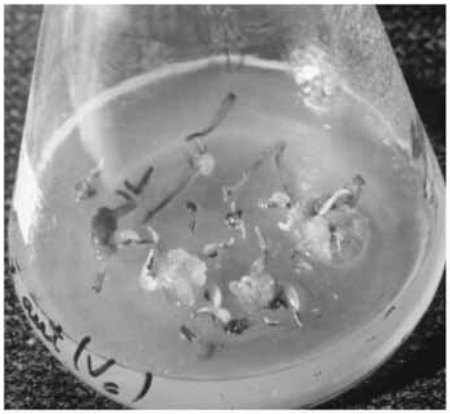


Fig. 1 Green friable callus from anthers-Cabbage

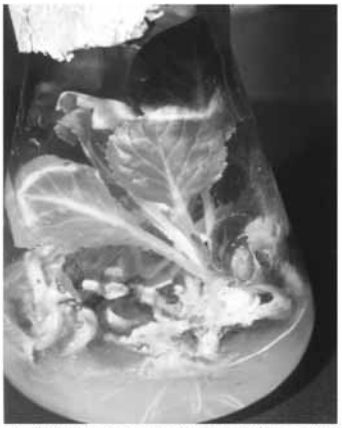


Fig. 2. Shoots regenerated on differentiation medium

Fig. 3. Neoplantlets generated from anthers of white cabbage (Z_2-12)

Fig 4. Green friable callus from anthers- Broccoli

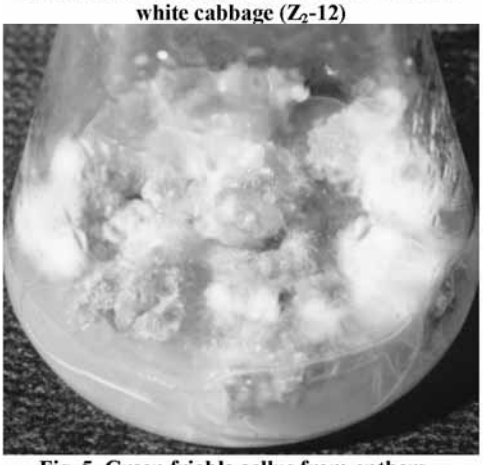


Fig. 5. Green friable callus from anthers – Cauliflower

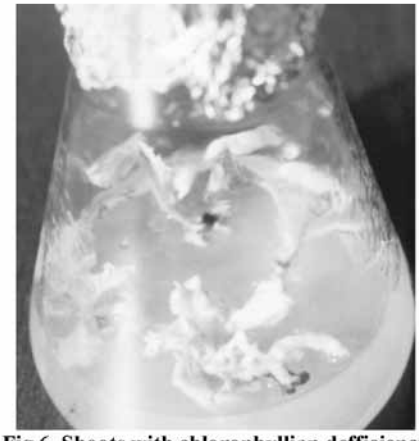


Fig.6. Shoots with chlorophyllian defficiency

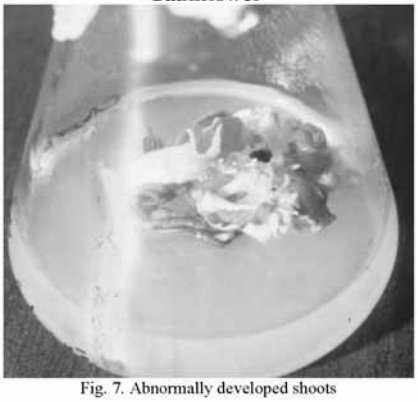


Fig. 7. Abnormally developed shoots

Fig. 8. Accommodated plants in soil pots

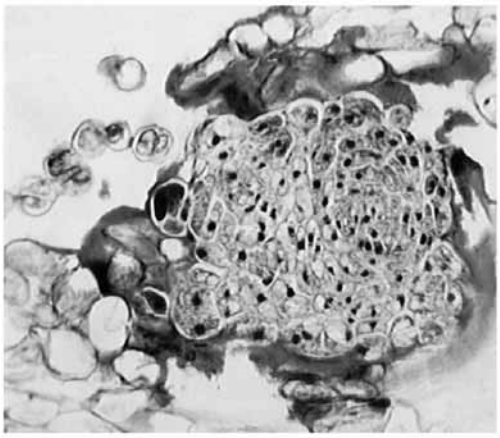


Fig. 9 – Meristematic centre

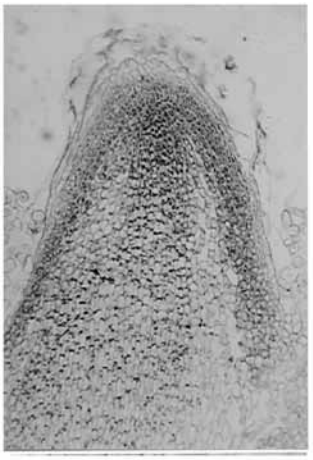


Fig. 10 – Root

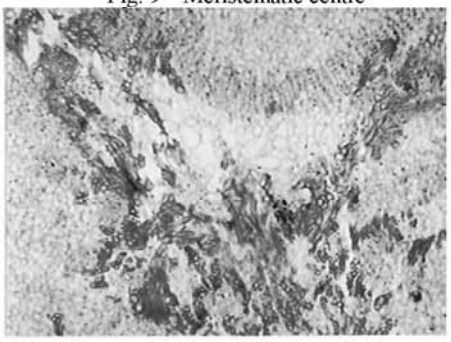


Fig. 11 – Tracheides from callus

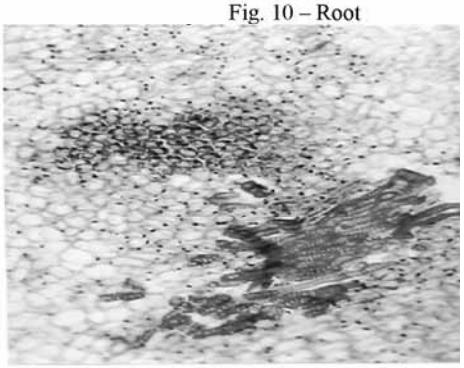


Fig. 12 – Tracheides and meristematic centre from callus

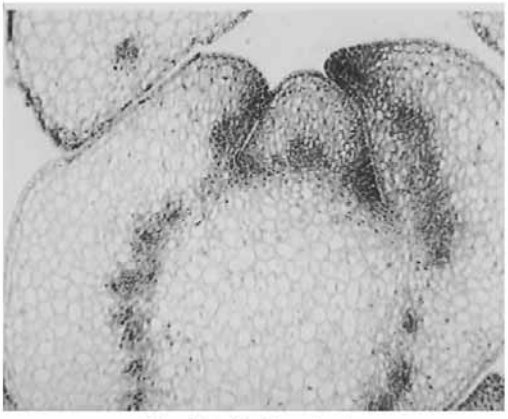


Fig. 13 –Axillary bud

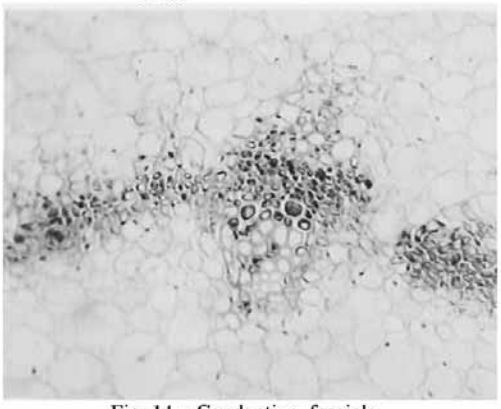


Fig. 14 – Conductive fascicle