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STUDIES ON THE IN VITRO BEHAVIOR OF THE OVARIES OF *BRASSICA OLERACEA* VAR. *BOTRYTIS*

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INTRODUCTION

Numerous wild forms of the species *Brassica oleracea* L. are found even today in the Mediterranean Sea area. Long before our era, the forms of this species were cultivated for their medicinal properties which were mentioned in old Greek and Roman writings. Today, the vegetables in the cabage group are frequently used in human and animal food, on account of the fact that they provide variation in the dietary regime and ensure the organism normal functioning, protecting it against serious diseases and early aging.

This „vitamin industry" contributes to vegetables having special *alimentary importance*, depending on their nutritional composition and amount consumed (PÂRVU C., 2000). *Broccoli* originates in the neighbouring area of the Mediterranean sea, being cultivated for the primordia of its blossom, sometimes elongated as terminal shoots. They are consumed cooked under different forms.

The economic importance of some species of the *Brassica genus* has drawn researchers'attention worldwide. Obtaining some valuable species through hibridation is intensively used, but requires long time and very hard work (GHIORGHIȚĂ G., NICUȚĂ-PETRESCU D., 2005). Scientific literature has reported numerous results about "in vitro" microspore cultures for species of the *Brassica* genus (DIAS J. C., 1999, ACHAR P. N., 2002, HU H., YANG H. J., 1986, KELLER W. A., 1984, NICUȚĂ D., GHIORGHIȚĂ I.G, MIHU G., 2003), whereas no data have been registered on ovary culture. Ovary culture has been initially practised with a view to clarifying some aspects such as morphogenetic, physiological and biochemical modifications of the fruit. Thus, the first ovary culture to this purpose has been reported by LA RUE (1942, citated by RANGAN, 1982), who registered a limited growth of the ovaries, accompanied by the rooting of the pedicle at more species. NITSCH (1951) has further expanded this technique to the physiological study of the fruit.

MATERIAL AND METHODS

Our researches used as biological material floral buttons harvested from four genotypes belonging to the species *Brassica oleraceea* L., variety *botrytis*, subvariety *cymosa*.

The blossom samples were taken from the greenhouse of the Vegetable Research and Development Station Bacau (VRDS Bacau); the experiments were made on the following genotypes: BR-312-3; BR-312-5; BR-11-2;BR-S.

The biological material was harvested during the period February to June inclusively, when donor plants were in the pre-flowering stage (2-3 days before flowering). The average of the growth temperature of mother plants was around 12^0C in February-March and 22^0 C in May-June.

Depending on the harvest and inoculation periods for the biological material, we found a different pattern of evolution of the phytoinoculs, both concerning their response to different medium variants and their reaction speed.

The moment the microspore or embryo sac, changes its normal gametophytic evolution to gynogenesis, respectively androgenesis, is well established, differing from one species to another or from one variety to another within the same species.

This is why we have collected floral buttons of different size, ranging between 4 and 6 mm, to reach the optimal stage for gynogenesis induction. From this point of view, we have noticed that, depending on the size of the flower button, the ovaries had the best reaction when they came from larger buttons (5-6 mm).

The biological material, represented by immature blossoms, whose floral buttons measured between 4 and 6 mm, was harvested and kept in a refrigerator for 24 hours.

The sterilization of the biological material was performed with 0.1 ‰ mercuric chloride solution for 10 minutes, followed by three repeated washings with sterile distilled water.

After sterilization, the ovaries were excised under a binocular microscope or with naked eye and then immediately placed on the initiation media of the invitro culture.

To test the morphogenetic response, the ovaries were inoculated on 8 hormonal variants on nutritional media. The MS solution was used as a basal medium (Murashige - Skoog, 1962).

The amount of sucrose introduced was 100 g/l and was solidified with agar nutrient solutions - 8gr/l. The type and concentration of plant hormones introduced in the initiation media are presented in table 1. Before autoclaving, the pH of the media was adjusted to 5.8.

Table 1. Hormonal variants used in preparing the initiation media

No	Hormonal formula	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	-

The calli derived from the initial media were transferred to the differentiation medium. For their preparation, we have used as basal medium a solution of mineral salts and MS vitamins (Murashige - Skoog, 1962), which reduced the amount of sucrose to 30%.

For the induction of callus differentiation, we have created several hormonal variants, for which we mainly used phytohormones of the class of citokinins in different concentrations (table 2).

Table 2. Nutritive media used for discriminating calli from ovaries

No	Basal medium	BAP mg/l	KIN mg/l	GA3 mg/l	Saccharose g/l	Agar g/l	PH
1.	MS	0,5	-	-	30	8	5,8
2.	MS	1	-	-	30	8	5,8
3.	MS	2	-	-	30	8	5,8
4.	MS	0,5	0,5	-	30	8	5,8
5.	MS	1	-	0,1	30	8	5,8

Shoots obtained directly from ovaries or callus from the ovaries were transferred to media that induce rhysogenesis. To this end, we used a variant containing only the basic MS nutrient solution (Murashige-Skoog, 1962) without hormones and two other variants to which low concentrations of NAA were added (table 3).

Table 3. Nutritive media for rhysogenesis induction

No	Basal medium	NAA	Saccharose g/l	Agar g/l	pH
1.	MS	-	30	8	5,8
2.	MS	0,5	30	8	5,8
3.	MS	0,7	30	8	5,8

The ovaries were excised in sterile conditions (at the laminar flow bench) and then inoculated into nutrient media, depending on the size of the floral button. Seeded pots were transferred into the growth chamber at a light intensity of approx. 2500-lux, photoperiod of 16 hours a day) and a temperature of 23-24^{0}C.To accommodate the new plantules of gynogenetic origin, we have used the hydroponic culture system. After approx. 10-12 days, the in vitro plants survived the septic environment.

The following stage was the transfer of the regenerants to pots with soil mixed with perlite, and when the plants proved to be vigorous enough, they were transplanted to the greenhouse of the VRDS Bacau.

RESULTS AND DISCUSSIONS

The inoculation of the ovaries from broccoli genotypes on the initiation media led to their growth in size, the appearance of the callus, the regeneration of shoots *via callus* or the shoot formation directly from explants (photo 2 and 3). Depending on genotype and the phytohormone combination from the callus initiation media, the callus generated by the ovaries had two consistency types, namely: brittle (granular) (photo 2) and compact (photo 1). The callus also varied in color. From this point of view, we have observed that the friable callus was of lime-green and white-cream color, whereas the compact one was green and cream.

The inoculation of the ovaries on the initiation medium which contained phytohormones of the auxin class (NAA, 0, 1 mg/l and 2,4-D – 0.1 mg/l) resulted in the generation of brittle, cream-white callus (fig. 1) with a high proliferation rate in the ovaries of the BR-312 BR-312-3-5 genotypes. For the BR-11-2 genotype, the ovaries generated small friable cream-colored callus from which, after about a

month, long roots were formed without side branching.

The same type of response was received for the ovaries of genotypes S and BR-BR-11-2, inoculated on the BAD variant (BAP-1 mg/l, IAA - 0.1 mg/l, 2,4 - D-0.5 mg / l). This combination of phytohormones has allowed high callus proliferation. Sporadically, for some calli, short root tufts were found covered with absorbent hairs (photo 2). This type of callus showed no caulogenetic potential, its proliferation rate decreased, and, in time, it browned and degenerated.

At the level of the ovaries of genotype BR-312-5, cultured on BAD version, we have observed the emergence of a dark green compact type of callus, which has developed strongly in the contact area of the ovaries with nutrient medium (fig. 2). Two weeks after the appearance of the callus, a few short and thick roots sprung from it and inserted into the nutritional medium. Transferred to a new medium, but with the same combination of phytohormones, the callus showed low proliferation, and continued to grow roots. The presence of the citokinins on the differentiation media could not influence the caulogenetic capacity of the callus cells. On these nutritional variants, the callus ceased to proliferate and degenerated with time.

The combination of BAP (1 mg/l), with 2,4-D (0.5 mg/l) and BAP (1 mg/l) and IAA (0.1 mg/l), gave rise to a semi-compact green callus (fig. 2). On these nutritional variants, the callus formation occurred on the entire surface of the explant. The proliferation rate was high for genotype-3 and BR-312 BR-312-5 and relatively good for BR-S genotype. Sporadically, out of the mass of some calli, there differentiated a few feeble roots, as well as shoot buds. The presence of the citokinins on the differentiation media has allowed the differentiation of shoots, the emergence of new ones, but also a more intense rhysogenesis.

The presence in the initiation medium of the BDN phytohormonal combination induced the emergence at about two weeks after inoculation of the lime-green brittle callus (fig. 3). This has been shown for the ovaries of genotypes BR-312-3-5 BR-312 BR-S and was characterized by a relatively good growth rate. At the level of the callus cells, there appeared caulogenetic islands that differentiated vigorous shoots on the initiation media.

The same type of callus was generated on the BN variant by the ovaries of the four genotypes tested. The callusogenetic intensity was high, showing a very good callus proliferation. Its subculture on a fresh medium with the same combination of phytohormones (BN) has led to the production of new masses of regenerative callus. Some calli gave shoots with abnormal growth (leaves much increased in size).

Green brittle callus was transferred to citokinin variants to the purpose of differentiation. After about 10-14 days, in the callus mass, there appeared small shoots across the entire surface of the callus. In the presence of high concentrations of citokinin, the shoots (with 2-3 basal branches) strongly developed. On the basal leaves, which were thick and large, there emerged small tufts of 3-4 new shoots.

In transferring both callus and shoots on variants B_2, BK and even on $B_{0,5}$ we have discovered a strong regenerative capacity, represented by the explosive emergence of new shoots both at the surface and in the nutritional medium (photo 3 and 4).

On the BDN and BN variants, a reduced number of ovaries were obtained by direct caulogenesis. Such a reaction has been found in genotypes BR-11-2 and BR-312-5 on the BDN medium, and at the BR-S genotype, on the BN medium. Shoots obtained on BN had leaves with chlorophyll deficiencies.

The combination of phytohormones (kin, 1mg/l 2,4-D, 1mg/l) resulted in the ovaries genotypes BR-11 BR-S-2 generating cream-colored callus, of hard consistency, characterized by a relatively good rate of proliferation. This type of callus showed no organogenetic capacity. Transferred to differentiation media, it has generated no shoots or roots.

For the differentiation of calli derived from ovaries on different hormone formula, we used nutritional media in which only citokinin was used of the phytohormone category. The induction of caulogenesis was best represented by B_2 and BK variants. On the variants with a reduced quantity of citokinins, only the green brittle callus differentiated shoots, but their number was reduced.

The rooting of shoots was achieved without problems by transferring the shoots on the MS medium without hormones. In approx. 2 weeks, most of the shoots had a strong root system, consisting of 3-4 roots. It is interesting that, in the absence of growth regulators on the MS medium, at the base of some shoots, new ones emerged (multiple shoot formation), so that newly-formed roots did not belong to a single shoot but to a tuft of 4-5 shoots.

The accommodating of the new plantules was achieved without problems within approx. 2 weeks.

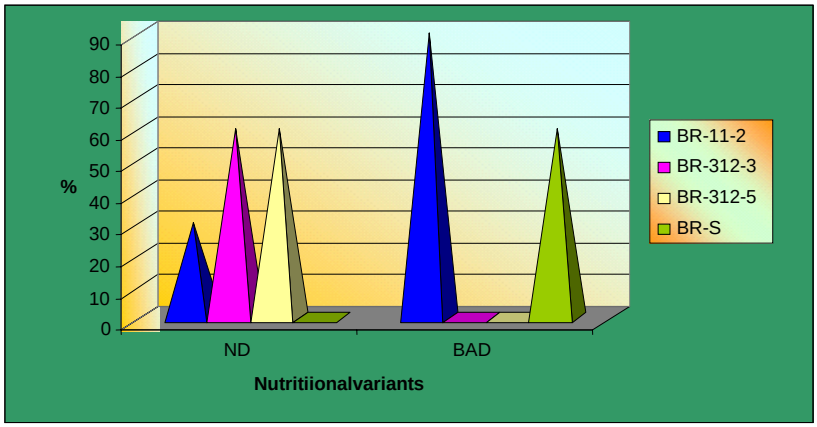

Fig. 1. The proliferation intensity of the cream-colored brittle callus from the broccoli ovaries to the initiation media

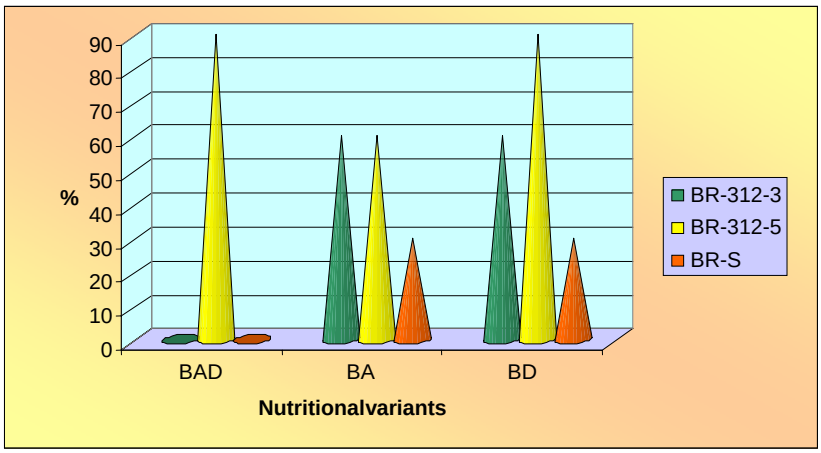

Fig. 2. The proliferation intensity of the green compact callus from the broccoli ovaries to the initiation media

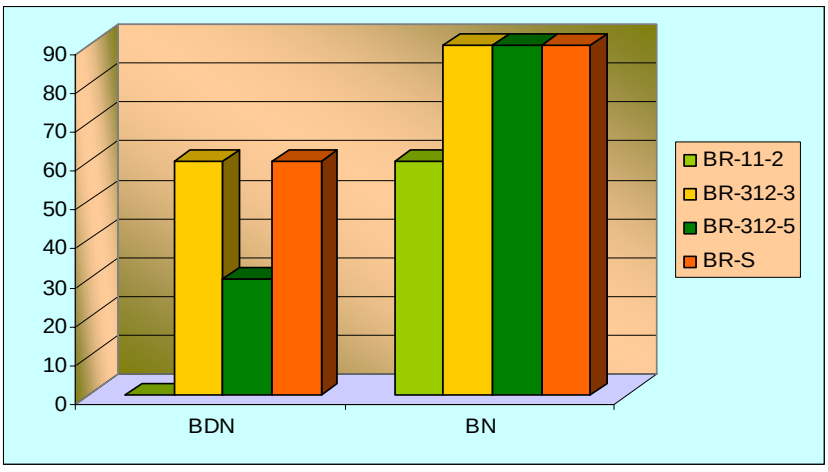

Fig. 3. The proliferation intensity of the green brittle callus from the ovaries to BDN and BN variants of *B. oleracea*, var. *botrytis*

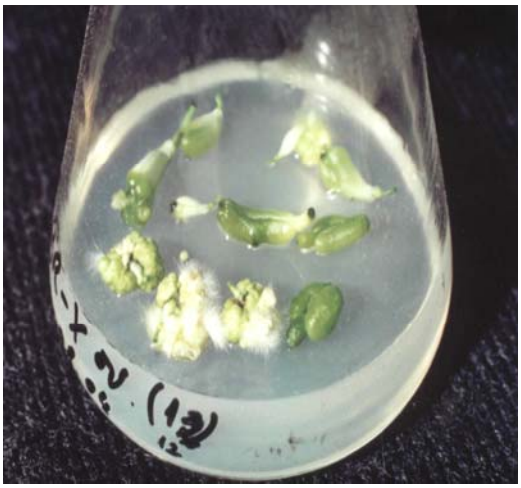

Photo 1. Enlarged ovaries and green compact rhizogen callus, from ovaries, on BD medium

Photo 2. Brittle callus from ovaries (BR-312-5), on BN medium

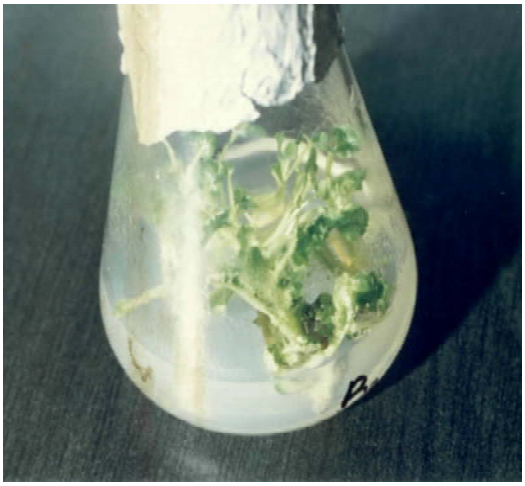

Photo 3. Multiple shoots from ovary callus on B_2 medium

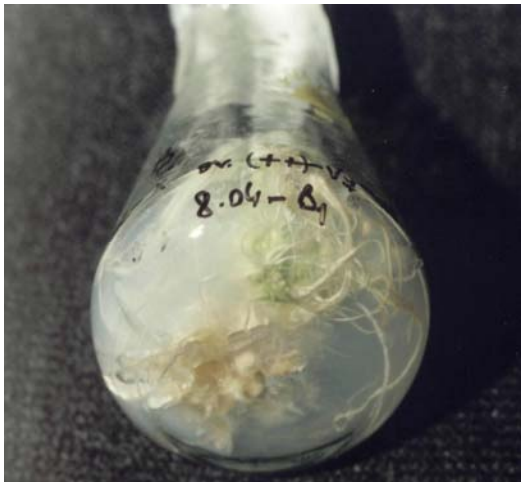

Photo 4. Callus from ovaries with roots and very small shoots on B_1 medium

CONCLUSIONS

- The morphogenetic response of the broccoli ovaries varied according to genotype, size and combination of phytoinoculs from nutritional media.
- At the level of the explants, there were found two types of reaction: the appearance of the brittle or compact callus and the sprouting of shoots through direct organogenesis;
- The friable cream-colored callus type was generated by inoculated ovaries on the BAD and ND variants. It was characterized by an average proliferation speed. The best reaction of this type was characteristic of the ovaries of BR-11-2 genotype on the BAD medium;
- Some ovaries inoculated on the BN and BDN media generated brittle green callus type. The proliferation rate was higher on the BN medium. A good callusogenetic response was given by ovaries of the genotypes BR-BR-312-3 and 312-5. The brittle callus was characterized by high caulogenetic capacity;
- The green compact callus was generated on the BAD, BA and BD media. Ovaries taken from the BR-312-5 genotype manifested the best callusogenetic response of this type, followed by those of genotype BR-312-3 and BR-S.
- The compact callus generated sporadic roots and a few shoots. On the differentiation media with high concentration in citokinin, shoots and roots were regenerated shoots in a very low percentage, but the rhysogenesis was much more intense;

- The emergence of shoots directly from the ovaries has been evidenced on BDN and BN open environments, but the phenomenon has been mild. A small percentage of chlorophyll deficiencies in shoots.
- The rooting of shoots was achieved on the MS medium without growth regulators.
- Vitroplants have been accommodated without problems in the hydroponic culture system and within two weeks they were transferred to pots with soil.

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ABSTRACT

The study of the in vitro behavior of broccoli ovaries was conducted using as biological material four genotypes, harvested from VRDS Bacau. The response of the ovaries inoculated on 8 nutritional variants was characterized by two types of reaction: the generation of compact and brittle calli and the emergence of shoots by direct organogenesis. The gynogenetic callus emergence, its consistency, color and regenerative capacity depended on genotype, hormone formula of the nutritional media and size. O the BN and BDN media the ovaries of the BR-312-3 and BR-312-5 genotypes generated green brittle callus, characterized by high caulogenetic capacity. The emergence of shoots directly from the ovaries has been proven on the BDN and BN media, but the phenomenon had reduced intensity. The rooting of shoots was achieved on the MS medium without growth regulators. In vitro plants have been accommodated without problems in the hydroponic culture system.

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