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CYTOGENETIC OBSERVATIONS ON SOME *IN VITRO* REGENERANTS PROVIDED BY OVARIES OF *BRASSICA OLERACEA* L. VAR. *CAPITATA*

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Abstract: The cytogenetic studies on *in vitro*-derived plants (by means of direct or indirect gynogenesis) – 6 genotypes of *Brassica oleracea* L. var. *capitata*, aimed to evince if and to what extent this type of conventional culture altered the mitotic cell division. There were some differences regarding the mitotic index, the distribution of cells on each phase of mitosis, and also on the percentage of abnormal ana-telophases. The gynogenetic shoots ($Z_{2\text{-}12}$ genotype) obtained from callus on a culture medium supplemented with 1 ml^{-1} BAP and 0.1 ml^{-1} IAA displayed the highest mitotic index (51.74%), compared to the control (18.10%) and to the other studied vitroplants. The cytogenetic analysis of the shoots provided by direct organogenesis on several hormone variants ($Z_{2\text{-}12}$ and TRM_1 genotypes) evinced a decrease of the mitotic activity, compared to the control (18.10%). Regarding the cell distribution on mitotic phases, the highest percentage was registered by prophases, followed by metaphases, telophases and anaphases (in all the analyzed variants, including the control). The cytogenetic studies displayed a rather similar and high percentage of abnormal ana-telophases. The frequency of chromosomal abnormalities was significantly influenced by the presence of growth regulators in the culture medium and also by the genotype.
Keywords: *Brassica oleracea*, gynogenesis, mitotic index, chromosomal aberrations.

Introduction

Brassica L. genus comprises a large range of diploid and amphiploid species, of which some are largely used as food plants. *Brassica oleracea* L. is a diploid species with 2n=18 chromosomes. *Brassica* chromosomes are difficult to study under a normal microscope, being very small and weakly differentiated (Hasterok and Maluszynska, 2000; Snowdon, 2007). At the beginning of the twentieth century, Morinaga (1928 - 1934) and U (1935) have established the chormosomal relations between diploid species of *Brassica* genus and their natural amphiploids (quoted by Ahmad et al., 2002; Snowdon, 2007). Based on the length and the centromere position, *Brassica* sp. chromosomes were grouped in 6-7 types (Richharia, 1937, cited by Ahmad et al., 2002).

The cytogenetic studies of haploids (Hosemans and Bossoutrot, 1983; Howell et al., 2002) have contributed the understanding some of the genetic mechanisms that explain the species evolution.

Through the cultivation of ovules and unfertilized eggs on artificial media (*experimental gynogenesis*), haploids can be obtained – *plants with no father* (Dunwell, 1985; Hu and Yang, 1986; Ferrie and Keller, 1995; Nicuţă et al., 2003). According to literature - based data, experimental androgenesis was achieved for over 170 species, while gynogenetic plants were obtained in only for species (Reed, 2005).

Gametoclonal variation refers to the variation observed in certain plants regenerated from gametic cell cultures and has been observed in many species. Gametoclonal variation

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may appear as numeric changes of chromosomes (polyploidy or aneuploidy). If for plants regenerated from anthers, a high percentage is represented by chromosomal variations, in the case of gynogenetic plants, this phenomenon has not been studied on a large scale (Reed, 2005).

The method of *in vitro* culture can be a source of genetic variation without the intervention of mutagenic agents (D'Amato, 1986). If plants regenerated *in vitro* from cultures of callus, cells, microspores, protoplasts etc, would manifest the same rate of genomic modifications, they would represent a source of variability and contribute to plant amelioration (Larkin and Scowcroft, 1981; Badea and Săndulescu, 2001).

Chromosomal instability is a feature of *in vitro* cultures of plant cells, which causes genotypic, and implicitly, phenotypic changes in regenerants (Duncan, 1997). Based on the observations made by various authors who have studied chromosome variations for *in vitro* plant cultures and their causes, Lee and Phillips (1988) concluded that: some variations pre-exist in explant; the explant type used in culture and the genotype may be an important source of chromosomal variation; the cytological state of the cells grown depends on the cultivation regime; chromosomal variation increases with the maintenance of *in vitro* culture; the disorganised culture of callus is more frequently associated with chromosome instability; transposable elements can be activated by *in vitro* culture, etc.

Some of the internal mechanisms of the genetic processes that lead to the occurrence of somaclonal variations have special relevance: polyploidy and aneuploidy, chromosomal restructuring, somatic crossing-over, exchange between sister chromatids (SCES), mobile genetic elements, genic amplification etc. (Raicu, 1990).

Along with numeric chromosomal changes of *in vitro* cultures and even more frequently than these, there are changes in chromosome structure. The phenomena of chromosome breakage and reunion in atypical configurations, together with the loss of genetic material, appear frequently in *in vitro* cultures and bring their contribution to somaclonal variation (Ghiorghiţă, 1992). These can cause the loss of some genes or the abnormal functioning of others, and even silencing gene expression etc. Badea and Raicu (1984) claim that gene loss due to chromosomal restructuring cancels their function, but can also trigger the activation of other genes, which, normally, are not functional.

During differentiation, organogenesis, embryogenesis and development processes, a cellular selection process and genotypes with a specific (normal) number of chromosomes are favoured to such an extent that regenerated plants are still diploid (Evans and Reed, 1981; Nuti Ronchi et. al., 1990).

Plant regeneration under *in vitro* culture conditions is determined by the cumulative effect of internal and external factors (genotype, nutritive media, culture conditions, etc). All these greatly influence the influence the explant morphogenesis, the speed of cell proliferation, as well as the occurrence and frequency of some chromosomal aberrations at different stages of mitotic division (Ghiorghiţă and Nicuţă - Petrescu, 2005).

Materials and methods

Cytogenetic studies have been conducted on roots of 1.5 to 3 cm in length, harvested from vitroplants obtained by direct gynogenesis on different nutritive media variants of MS (Murashige – Skoog, 1962) (Table 1), 6 genotypes of *Brassica oleracea* L.

convar. *capitata* (L.) Alef. var. *capitata* ($Z_{2\text{-}12}$, TRM_1, DE, BCO-076, BCO-7-10, RM_1) were used.

The control variant used comprised small roots obtained from seeds belonging to the same species.

Table 1. Hormonal variants which induced gynogenetic regenerants through direct organogenesis or via callus in *Brassica oleracea* L. var. *capitata*

Crt. No.	Hormonal formula	BASE MEDIUM	GROWTH REGULATORS (mg/l)				
			IAA	IBA	NAA	2.4-D	BAP
1.	BB_2	MS	-	0.1	-	-	1
2.	BD	MS	-	-	-	0.5	1
3.	BDN	MS	-	-	0.1	0.02	0.3
4.	BN	MS	-	-	0.1	-	0.5
5.	BA	MS	0.1	-	-	-	1

The plant material was fixed in Framer solution and hydrolysed with HCl 50%for 8 -10 minutes.

Colouring was achieved in a basic carbol-fuchsin solution, in concentration of 10%.

The slides were prepared using the "squash" technique.

Fresh materials have been examined under an optical microscope (NOVEX), exposed to intense light using a violet filter to highlight the contrast between chromosomes and cytoplasm. The photos of different phases of mitotic division have been taken using the 40x and 100x objectives, with an OLYMPUS digital camera.

The mitotic index (MI) was determined after the analysis of 50 microscopic fields/genotype. Also, the chromosomal aberrations were recorded.

Results and discussions

In our study, the morphogenetic evolution of the explants has resulted from the interaction of the following factors: genetic information existent in each explant, hormonal balance in nutritive media, and cultivation conditions (Nicuţă et al., 2003).

Considering that all these factors contribute to a certain extent to the phytoinocule evolution and to the genetic constitution of the future plants, it cannot be assessed exactly what determines the changes at the DNA level (polyploidy, aneuploidy, etc.) or what factor is essential for the disruption of cell division, which causes various types of chromosomal aberrations.

Due to the enormous genetic variability existing in each explant, the morphogenetic processes are determined by the following factors: phytohormone type, concentration of the nutritive media, the presence or absence of items required for growth, the quantity of oxygen in explants, the presence of substances with mutagenic effect released by explants in the nutritive media etc. The influence of phytohormones on the growth and development of plants in vitro can be found in other specialized publications. Thus, Selma and Signem, (2012) revealed the negative effect of abscisic acid (ABA) towards cytogenetic activity in root meristem cells of *Hordeum vulgare* L. Compared to the plants control group, the treatment of barley seeds only with ABA resulted in a significant decrease of mitotic index and at the same time the increase of chromosomal aberrations percentage. Instead,

combining ABA with other growth regulators induced an antagonic effect towards the same parameters investigated.

In vitro and in vivo researches to *Celosia cristata* L. plants have shown a mitotic index increased to in vitro regenerants compared to in vivo plants (Taha and Wafa, 2012).

The influence of genotype towards tolerance to salinity was revealed in researches on cell activity from root meristems at two cultivars of *Brassica napus* L. (Homa, 2007).

Therefore, the interpretation of results has been carried out taking into account the genotype and hormonal balance existing in each medium variant in which the regenerants have been obtained.

The study of microscopic material for gynogenetic regenerants of *Brassica oleracea* has revealed that root cells presented different forms – oval, spindly or rectangular, while on the control material, the cell form was generally the same (rectangular).

In terms of size, the cells in control plants were bigger, their observation being performed more easily (and with 40x objective). The material prepared from vitroplant roots has emphasized much more reduced sizes, sometimes their observation being difficult even under a 100x objective.

Mitotic index and frequency of mitotic division phases in callus and ovary-derived regenerants

For ovary culture, vitroplants have been obtained by (direct or indirect) organogenesis. Nutritive variants which favoured these phenomena included: BDN, BA, BD, BN and BB_2 (Table 1). The mitotic activity of the cells was seriously influenced by the genotype, phytohormone type and its concentration in the nutritive medium.

In $Z_{2\text{-}12}$ genotype, the regenerants of the BA hormonal variant have registered the highest mitotic index (51.74%), compared with the control sample and the other variants. By contrast, the regenerants of BD variants and BB_2 have registered more reduced values of the mitotic indices (15.85% and respectively 16.13%) (Fig. 1). Regarding cell distribution on division phases, the highest percentage belongs to prophase, followed by metaphase, telophase and anaphase in both control and experimental variants. Compared with the control plant, depending on the medium variant, the regenerants have registered increased cell frequency in metaphase (BB_2 – 14.0%) and anaphase (BN – 7.1%).

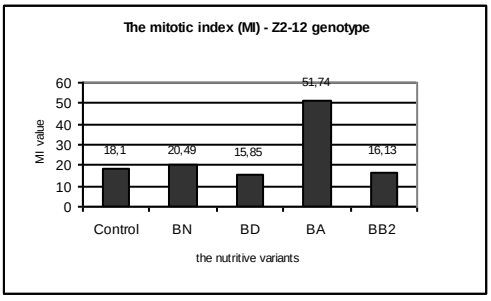


Figure. 1. The mitotic index of $Z_{2\text{-}12}$ genotype

Hormonal variants, on which vitroplants *via callus* of ovaries have been obtained in TRM_1 genotype, have induced a stimulation of MI and presented the highest value in BB_2 variant (49.24%) (Fig. 2). Cell frequency in division phases has decreased for BDN and

BB_2 variants, but increased for BN variant, compared with the control variant. In this particular case, the cell percentage in telophase is also higher than in anaphase.

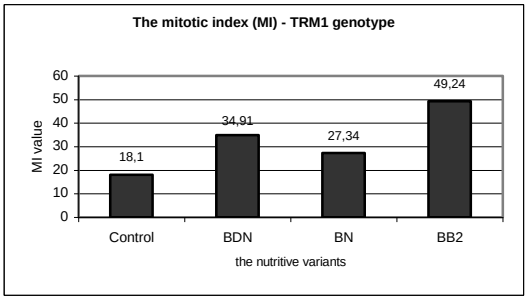


Figure 2. The mitotic index of TRM1genotype

DE regenerants which are gynogenetic and callus-derived, on BDN and BA variants, have presented a reduced mitotic activity, compared with the control sample (Fig. 3). The most reduced MI value has been calculated for BDN variant (15.38%). The analysis of cell distribution on division phases has underlined the predominance of prophases, followed by metaphases in all variants, including the control. For the control and BA variants, the number of cells in anaphase is smaller than in telophase.

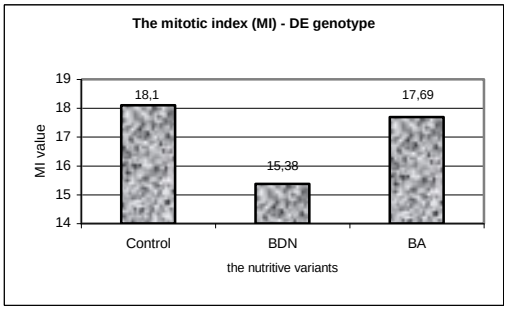


Figure 3. The mitotic index of DE genotype

In *BCO-076* genotype, MI value has increased compared with the control (18.10%), the highest value being registered on BA medium (37.96%). BDN variant has registered the smallest MI frequency – 14.20% (Fig. 4). Cell frequency in division has increased or decreased insignificantly compared to the control variant, depending on the medium variant. The maximum percentage of cell in mitosis has been registered in prophases, followed by metaphases, for all variants. For the control sample and the BDN, BD and BN variants, the percentage of cells in telophase has been much higher than in anaphase.

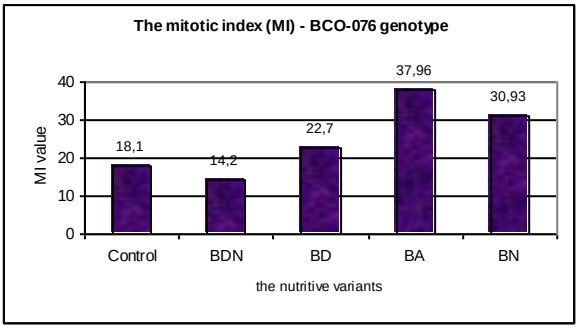


Figure 4. The mitotic index of BCO-076 genotype

The cytogenetic analysis of *BCO-7-10* vitroplants with gynogenetic origin has indicated a slight increase of the mitotic activity compared with the control sample. The highest MI value has been identified for BA variant, representing a 10% increase compared with the control variant (Fig. 5). *In vitro* culture conditions for the three hormonal variants have led to a growth in metaphase cell frequency, anaphase and telophase.

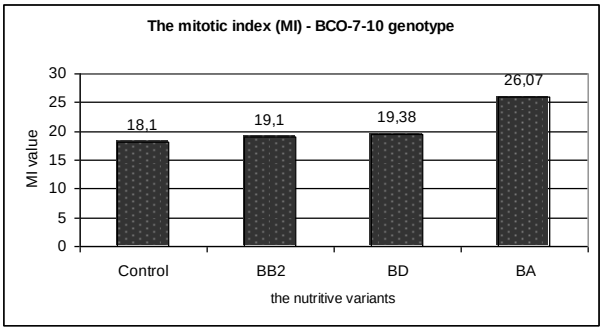


Figure 5. The mitotic index of BCO -7-10 genotype

For RM_1 genotype, the hormonal relationship of the culture media has induced the stimulation of the mitotic index both on the BDN and BN variants (Fig. 6). The highest level of MI stimulation has been indicated for BN variant. The maximum frequency of the cell stage division has been registered by prophases, followed by metaphases, telophases and anaphases. For RM_1 genotype, *in vitro* regenerants have registered an increased percentage of cells in metaphase and anaphase, compared with the control variant.

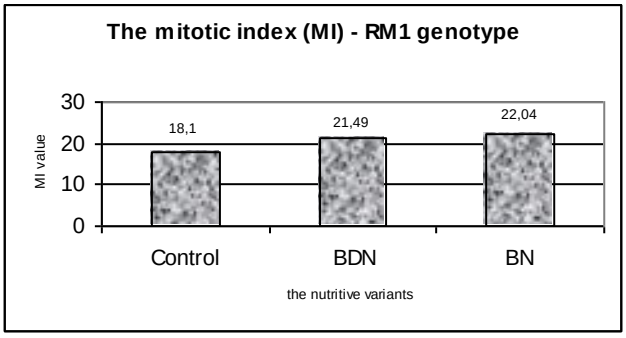


Figure 6. The mitotic index of RM1 genotype

The mitotic index and the frequency of mitotic division phases in regenerants derived from ovaries through direct organogenesis

Shoot regeneration directly from ovary explants has been observed in some nutritive variants only for $Z_{2\text{-}12}$ and TRM1 genotypes. In $Z_{2\text{-}12}$ genotype, the phytohormonal combination and *in vitro* culture conditions have triggered shoots growing through direct organogenesis. Cytogenetic studies have revealed a reduced mitotic activity on the two medium variants tested, compared with the control. BDN variant has registered the lowest MI (10.70%) (Fig. 7). For both control and experimental variants, the maximum frequency of cells in mitotic division has been calculated from prophases, followed by metaphases, telophases and anaphases.

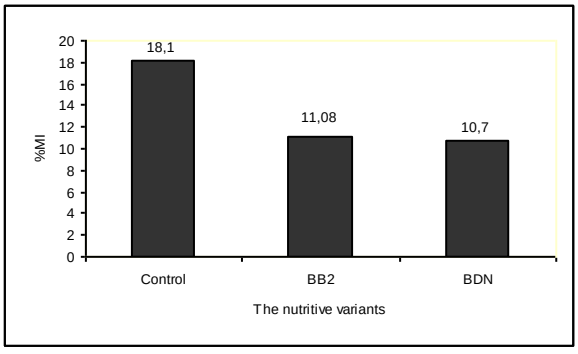


Figure 7. The mitotic index of $Z_{2\text{-}12}$ genotype – vitroplants obtained by direct organogenesis

In the case of TRM_1 genotype, regenerants have been obtained by direct ovary organogenesis only on BB_2 medium. These have registered a mitotic index equal to the one of the control variant. The maximum frequency of cell types in division corresponds to prophases; under both circumstances, there have been an increase percentage of metaphases, anaphases and telophases, compared with the control.

The study of aberrant ana-telophases in the mitosis of radicular meristems in *Brassica oleracea* regenerants derived from ovaries

The incidence of globular formations (pre-chromosomal) was highlighted by Li Xun (1999) for rape (*Brassica napus*). Studying the formation of mitotic chromosomes, the author indicated the presence of numerous weakly or more intensely coloured granules of different sizes in the nucleus. The photon microscopy analysis revealed that they are composed of heterochromatin and are linked together by intergranular fibrils. For this reason, some grains have an elongated fusiform aspect.

On our cytogenetic material, some cells presented filiform chromosomes (the central area is slightly thicker than their terminal region), which also seemed linked.

Li and Guan (1999) states that darker granulations have merged through intergranular fibers, which contribute to the formation of filiform chromosomes. Granulations disappear with the maturation of prophases and the emergence of chromosomes in the nucleus. It seems that these grains begin to form as early as the interphase. The same author claims that, in the chromosome formation process, aberrations do occurs, which seems to be confirmed by our observations, as far as the frequency of chromosomal aberrations in the mitosis of studied genotypes is quite high. The slightly brittle filiform chromosomes and the biochemical changes occurring in cells are factors favoring abnormalities in chromosome replication. As a result of induction of micronuclei aberrations, there may occur micronuclei through the union of heterochromatin granules. On the cytogenetic material in the gynogenetic regenerants of *Brassica oleracea* species, there have been identified a few cells containing micronuclei. Their dimensions were reduced and their number per cell was greater than 1.

During the cytogenetic observations, different chromosomal aberrations have been evinced in the cell ana-telophase, but the most frequent have been the cells with bridges (Fig. 16), fragments and delayed chromosomes (Figs. 17, 18, 19, and 20).

The frequency of cytogenetic abnormalities in *Brassica* plants regenerated from ovaries on various nutritive media compared with control plants depends on the nature and genotype of cultivated explants, as well as on the culture medium supplemented with certain hormonal formula.

When referring to more frequent types of chromosomal aberrations found in our observations, we can say that ana-telophases with bridges - (B) have been most numerous both in control plants (68.1%) and gynogenetic ones. The percentage was up to 88.1% in DE genotype for vitroplants regenerated on the BA nutritive variant (Fig. 9). The lowest frequency of this type of chromosomal aberrations has been calculated for genotype BCO-076, obtained on BD medium (Fig. 13).

In this species, an important percentage of aberrations are represented by delayed chromosomes - (DC), even in control plants (18.1%). In general, the increased ratio of regenerants obtained on various hormonal formulae tested is relative to control plants, reaching between 20 and 53.8% of the total number of aberrant ana-telophases identified. The highest value of this type of aberration occurred in genotype BCO-076, where the ovaries were cultivated on BN (53.8% of total aberrations) (Fig. 13). There are also variants in which the percentage of cells with delayed chromosomes (DC) is lower than in the control plant; cabbage genotype indicated only 5.8% of the total BA medium (Fig. 9) and BCO-7-10 a percentage of 10.2% out of all aberrations on BB2 (Fig. 12). Similar situations

have been found in the percentage of fragments (F) from all registered aberrations. Thus, on some hormonal formulae, plants regenerated from ovaries of some genotypes reached values of fragments of 33.3% (genotypes Z2-12 - Fig. 8 and BCO-076 - Fig. 13, grown on BD), or even 66.7% of total aberrations in RM1 genotype on BDN (Fig. 11). Obviously, in such cases, the percentage of cells with bridges or delayed chromosomes shall be reduced accordingly. The lowest percentage of ana-telophases with fragments has been indicated in genotype Z2-12 (5.5%), for plants obtained on BN hormone variant, while for the control variant, the percentage was higher (13.6%) (Fig. 8). The overall results, showed that genetic instability has been induced by gynogenetic plants of TRM1 genotype (Fig. 10), followed by those of BCO-076 genotype (Fig. 13).

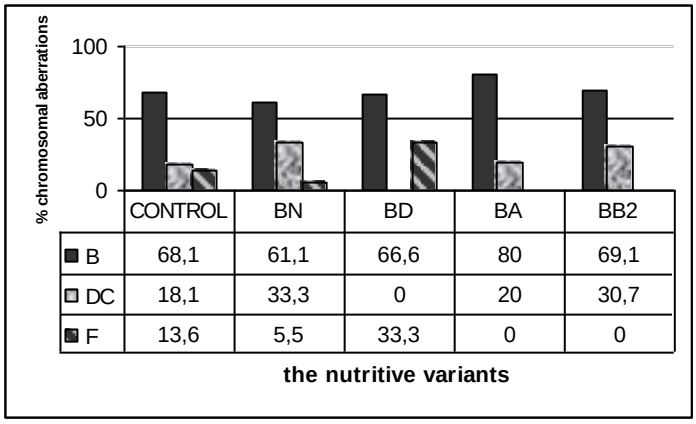


Figure 8. Frequency and spectrum of chromosomal aberrations identified in Z2-12 genotype; (B- bridges, DC- delayed chromosomes, F- fragments)

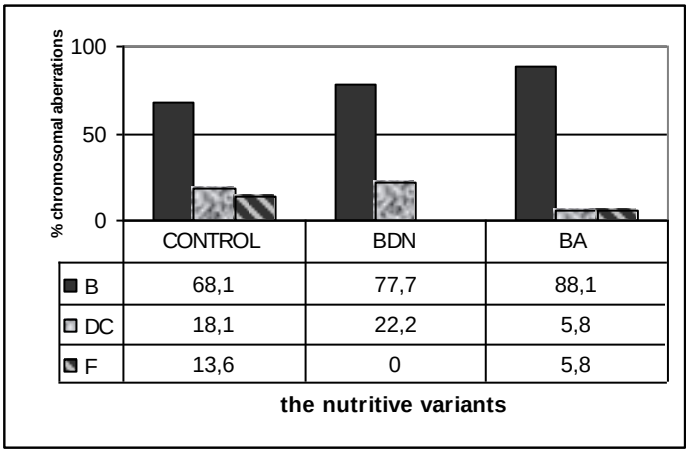
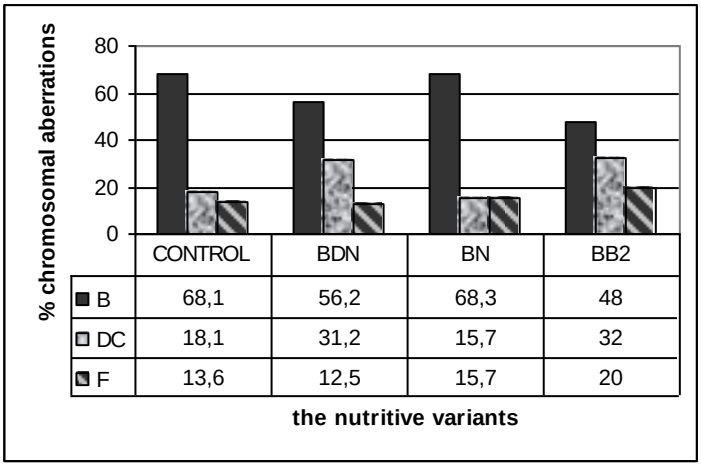
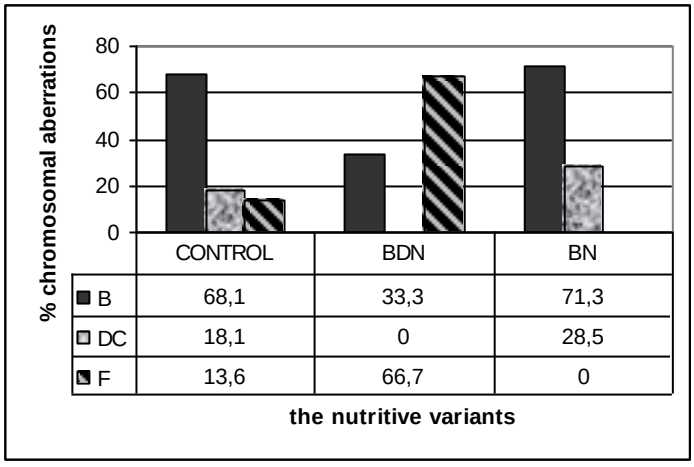


Figure 9. Frequency and spectrum of chromosomal aberrations identified in DE genotype; (B- bridges, DC- delayed chromosomes, F- fragments)



	CONTROL	BDN	BN	BB2
B	68,1	56,2	68,3	48
DC	18,1	31,2	15,7	32
F	13,6	12,5	15,7	20

Figure 10. Frequency and spectrum of chromosomal aberrations identified in TRM1genotype; (B- bridges, DC- delayed chromosomes, F- fragments)



	CONTROL	BDN	BN
B	68,1	33,3	71,3
DC	18,1	0	28,5
F	13,6	66,7	0

Figure 11. Frequency and spectrum of chromosomal aberrations identified in RM1 genotype; (B- bridges, DC- delayed chromosomes, F- fragments)

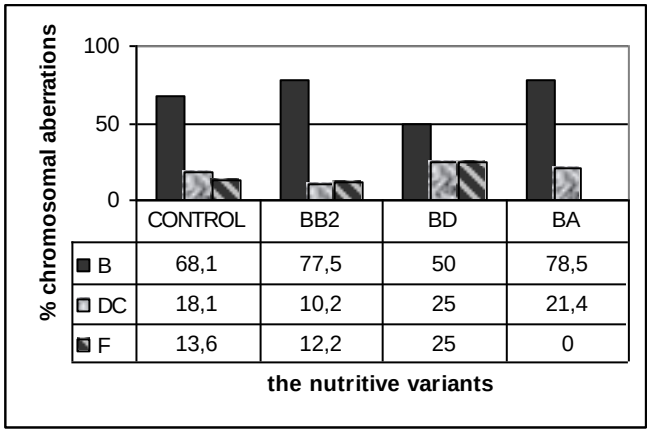


Figure 12. Frequency and spectrum of chromosomal aberrations identified in BCO-7-10 genotype; (B- bridges, DC- delayed chromosomes, F- fragments)

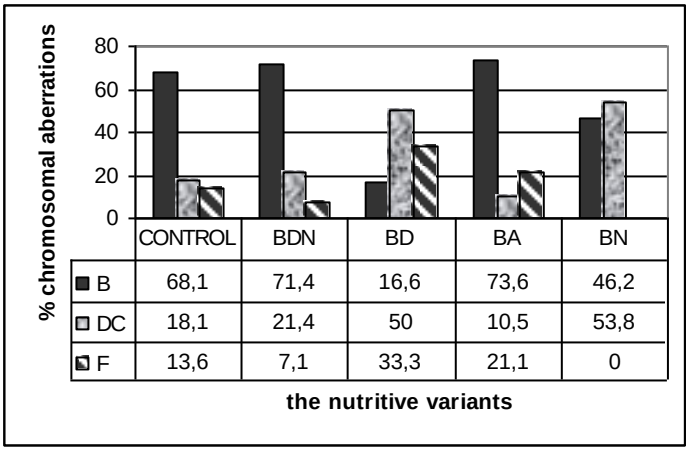


Figure 13. Frequency and spectrum of chromosomal aberrations identified in BCO-076 genotype; (B- bridges, DC- delayed chromosomes, F- fragments)

In *Brassica* plants derived from ovaries through direct organogenesis on different hormonal formulae (Z2-12 and TRM1), the frequency of aberrant cells in ana-telophase is much more reduced (between 5.3 and 6.3%) than in the control variant (11.5%). An explanation might be the fact that these plants have been regenerated directly from ovaries and not via callus which can increase the genetic variability of the regenerated material. The most significant types of aberrations signalled were bridges in both control variant (68.1%) and in vitro-derived plants (61.1%- TRM1 genotype and 75%- Z2-12 genotype), as well as delayed chromosomes (between 18.1% and 33.3% as compared to 18.1% in the control variant (Figs. 14 and 15).

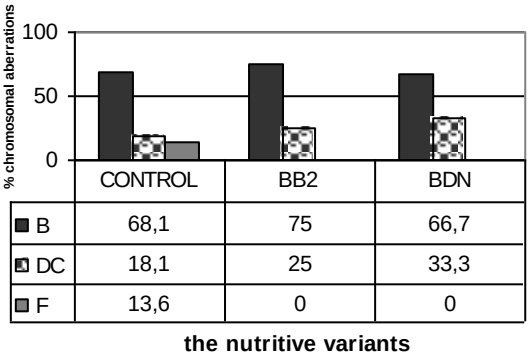


Figure 14. Frequency and spectrum of chromosomal aberrations identified in genotype Z2-12; vitroplants obtained by direct organogenesis

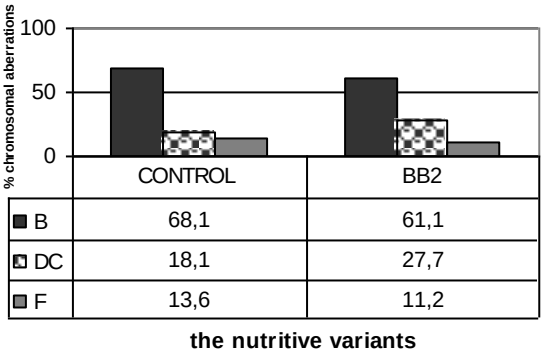


Figure 15. Frequency and spectrum of chromosomal aberrations identified in genotype TRM1; vitroplants obtained by direct organogenesis

Chromosomal aberrations have been identified in other phases of mitotic division, even in interphase. Thus, in some prophases (Figs. 21, 22), not all genetic material condenses to form chromosomes or the condensation in the nucleus is uneven, and some of it is already formed in a micronuclei. We have also identified metaphases (Figs. 23 and 24) with chromosomes which are expelled, or chromosome heterochromatic, fragments etc. It is rather difficult to find plausible explanations for the high incidence of chromosomal aberrations recorded in these species. It is possible that the packaging itself and the uneven condensation of nuclear material, the presence of large blocks of heterochromatins in prophase etc. might favour breakages, joining the ends of some chromosomes to form dicentric chromosomes, delaying the migration of chromosomes to the mitotic spindle during division etc.

This species appears to manifest naturally a high chromosomal instability. This can explain the great diversity of forms and varieties produced by artificial selection. This

chromosomal instability has been enhanced by in vitro cultivation and regeneration. Is this phenomenon due to the nature of explants, to the fact that plants were regenerated from callus of ovaries? As we have made cytogenetic observations on regenerants derived from other explant types, it is difficult to decide, but we tend to think that explant nature plays a role, besides growth regulators and stress provoked by *in vitro* culture itself.

We intend to further expand our cytogenetic studies on this species – on the one hand, in order to isolate haploid plants and on the other, to find plausible explanations regarding the chromosomal instability already observed.

Conclusions

1. The cytogenetic observations made on regenerants obtained from in vitro cultivation of *Brassica oleracea* ovaries have indicated that this system of cultivation does not disturb the functioning of the mitotic apparatus, i.e. there have been registered cells in all phases of division.

2. Mitotic activity of cells in the ovary-derived regenerants depended on genotype, explant type, hormonal balance of the nutrient medium, hormone ratio etc.

3. In regenerants obtained from ovaries, the distribution of cells on different phases of mitotic division does not change compared with control plants, the highest frequency being held by prophases followed by metaphases and telophases and the lowest in anaphases.

4. The frequency of aberrant ana-telophases and types of chromosomal aberrations depend on genotype, explant type and hormonal balance of nutritive medium.

5. There is a relatively high frequency and a relatively wide spectrum of chromosomal aberrations identified, even in control plants (derived from seed).

6. Of chromosomal aberrations identified on gynogenetic regenerants, the highest frequency has been registered by ana-telophases (A-T) with bridges, followed by A-T with delayed chromosomes and then A-T with fragments. The ratio of aberrations depended on the genotype and hormone formula of regenerated plants.

7. Microscopic observations have indicated A-T with complex aberrations

8. Cytogenetic abnormalities have been identified in prophase, metaphase and even in interphase.

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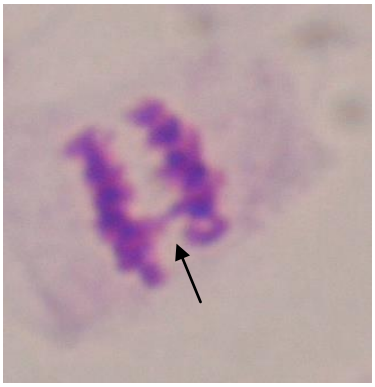

Figure 16. DE Genotype
Anaphase with bridge (100x)

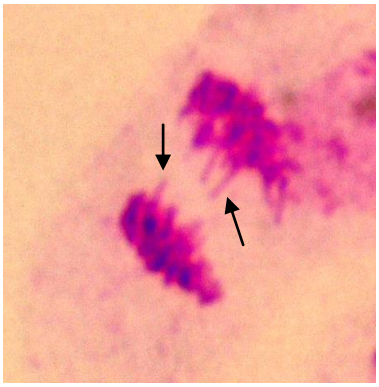

Figure 17. BCO-076 Genotype
Ana-telophase with delayed chromosomes (100x)

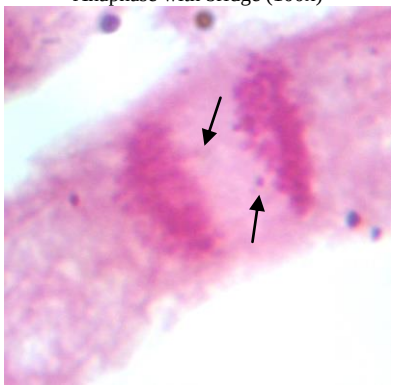

Figure 18. BCO-7-10 Genotype
Telophase with delayed chromosomes and fragment (100x)

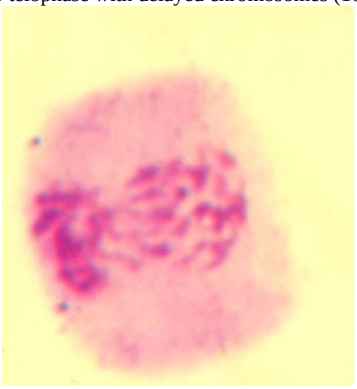

Figure 19. DE Genotype
Disorganized anaphase (100x)

Figure 20. BCO-7-10 Genotype
Telophase with fragment and expelled chromosom (100x)

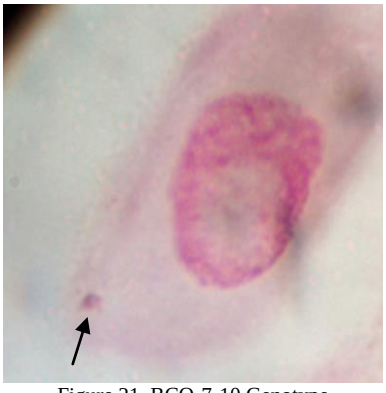

Figure 21. BCO-7-10 Genotype
Prophase with micronucleus (100x)

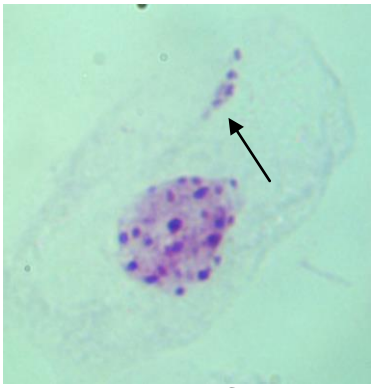

Figure 22. Z2-12 Genotype
Prophase with genetic material expelled (100x)

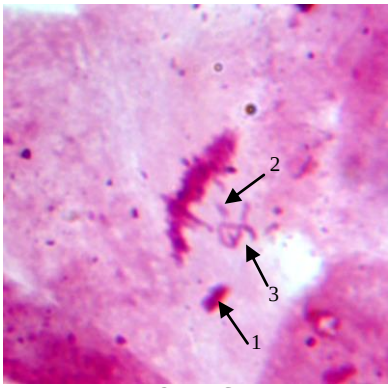

Figure 23. DE Genotype
Metaphase with chromosome heterochromatic (1), fragments (2) and delayed chromosomes (loop) (3), (100x)

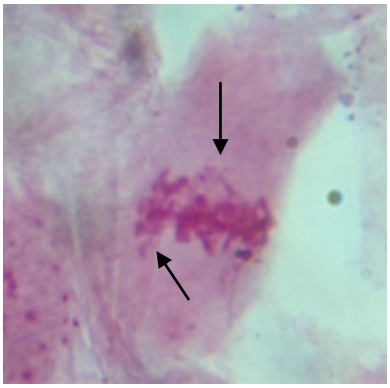

Figure 24. TRM_1Genotype
Metaphase cu expelled chromosomes (100x)

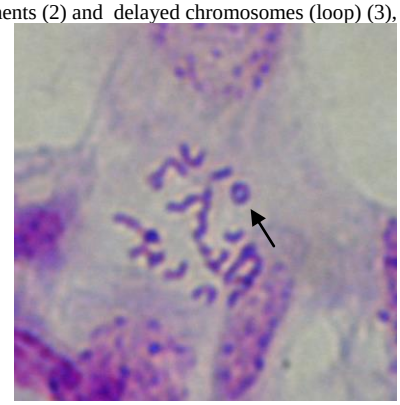

Figure 25. BCO-076 Genotype
Pro-metaphase with ring chromosom (100x)