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CYTOGENETIC OBSERVATIONS ON *IN VITRO* REGENERANTS OF *VERONICA OFFICINALIS L.*

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Key words: *Veronica officinalis, vitro-plants, mitotic index, chromosomal aberrations*

INTRODUCTION

Veronica officinalis, heath speedwell (common speedwell, gypsyweed, or Paul's betony) is a species of Veronica, belonging to Order Lamiales, Family Plantaginaceae. It is a dwarf herbaceous plant with hairy crawling stalk. It reaches up to 25-30 cm, and its bloom (with bluish flowers) points upwards, (CHIFU T., MÂNZU C., ZAMFIRESCU O., 2006; FISHER E., 2000). It is highly appreciated by German people.

In phytotherapy, heath speedwell is known as the "remedy against all evils", due to its content rich in active principles, of which bitter substances, heterosides, tannins, volatile oil, resins, glycosides etc. (STĂNESCU U. et al., 2002).

The tea made of it is recommended in different digestive and respiratory diseases, as well as in treatment of neurologic or circulatory system diseases (PÂRVU C., 2000).

The *in vitro* cultures of plants, used as methods of conservation and multiplication of valuable genotypes, suffered from variations, a phenomenon named by SIBI (1971), as pheno-variation or vitro-variation. These genetic modifications are transmitted to regenerated plants and their descendents, allowing their use for ameliorative programs. Among the factors influencing the emergence and frequency of somaclonal variations, there are: the growth medium, its supplementation with different hormones, some substances with mutagen effect, toxins and treatment with ionized radiations etc. (CORNEANU M., CORNEANU C., 2005).

There are registered phenomena of genome reorganization and its rapid evolution in the *in vitro* culture of vegetal cells and tissues, among which somaclonal variations are generated (BADEA E., SĂNDULESCU D., 2001; SNOWDON J. R., 2007).

The method of *in vitro* culture can be 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 genomic modifications reported at the same rate, they would ensure a huge source of variability and of forms useful for plant improvement (LARKIN and SCOWCROFT, 1981; BADEA and SANDULESCU, 2001).

Chromosomal instability is a feature of *in vitro* cultures of plant cells, which causes genotypic, and implicitly, phenotypic changes in regenerants. In synthesizing 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.

Cytogenetic studies on *in vitro* regenerates of different plant species emphasized the numeric chromosomal changes and the ones produced within chromosome structure (EARLE E.D., DEMARLY Y., 1978; RAICU P., BADEA M., STOIAN V., 1981; CORNEANU M., 1998). Thus, the inner mechanisms of genetic processes leading to somaclonal variations include: polyploidy and aneuploidy, chromosomal restructuring, somatic crossing-over, sister chromatid exchanges (*SCE*), mobile genetic elements, gene amplification, etc. (RAICU, 1990, CORNEANU M., 1998).

MATERIAL AND METHOD

The cytogenetic studies were conducted on radicular meristems of some *Veronica officinalis* L. regenerants. The vitro-plants were obtained by direct organogenesis from apical and modal explants, inoculated on 7 nutritive variant of the MS medium (Murashige –Skoog, 1962) (photo 1). In 6 variants were introduced growth regulators in different combinations and concentrations (Table 1), and for variant 7 – control – no phytohormones were used. The culture dishes were incubated in a SANNYO climatized room, in controlled temperature (22.5ºC) and light (16 hours photoperiod) conditions. The regenerated shoots were transferred on the MS

medium without phyto-hormonal supplements, for inducing rhizogenesis.

The sampling of roots for cytogenetic investigations was achieved after approx. 12 days since cultivation. The length of the samples roots ranged between 0.8 and 1.5 cm, (photo 2) .

Table 1. Nutritive variants used for the regeneration of *Veronica officinalis* L. vitro-plants

Crt .No	Hormonal formula	GROWTH REGULATORS (mg/l)					
		IAA	IBA	NAA	2,4-D	BAP	KIN
1.	MS (control variant)	-	-	-	-	-	-
2.	BB1	-	0,5	-	-	1	-
3.	BA1	0,1	-	-	-	1	-
4.	BN1	-	-	0,5	-	1	-
5.	KN	-	-	0,25	-	-	1
6.	A2	2	-	-	-	-	-
7.	N2	-	-	2	-	-	-

To the purpose of fixation to preserve the material, we have used a Framer solution and the hydrolysis process consisted of treating roots with a solution of HCl 50% for 8-10 minutes.

Coloring was achieved in a basic carbol-fuchsin solution, in concentration of 10% Carr-Walker reagent (Gamborg and Wetter, 1976).

The microscopic material was prepared using the "squash" technique.

Fresh materials have been examined under an optical microscope (NOVEX), exposed to intense light and 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.

In order to analyse the mitotic index (MI), there have been analysed sets of 50 microscopic fields for each genotype as variants of the nutritive medium. For chromosomal aberrations, the study involved observing all the normal and abnormal ana-telophases (A-T) per slide.

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.

The cytogenetic studies on the radicular meristems of regenerants obtained on 7 nutritive variants indicated that the mitotic index (MI) was high, depending on the hormonal formula of the nutritive medium. Thus, the lowest IM (30.70) was registered for vitro-plants obtained on the MS medium supplemented with 1mg/l BAP and 0.5 mg/l

For each nutritive variant, there were effected three repetitive inoculations of apical and nodal explants.

IBA, this value being more reduced compared with the mitotic index of the control variant (roots coming from regenerated vitro-plants on the MS medium without hormones) – 35.48 (Table 2).

The highest value of MI was calculated for regenerants obtained on the BA_1 variant (Table 2) – 45.63 and very close to it for regenerants obtained on A_2 (45.14).

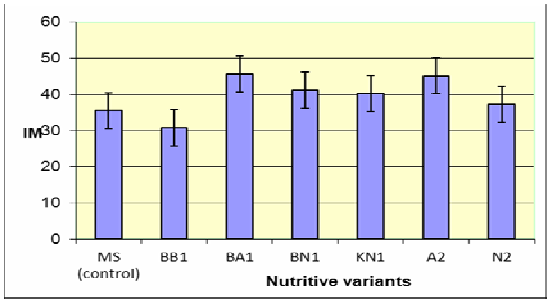

Fig 1. The mitotic index calculated *V. officinalis* L vitro-plants

Regarding the distribution of the phases of mitotic division, cell in prophase were predominant, followed metaphases, telophases and anaphases at the majority of the vitro-plants studied. For control regenerants, the percentage of anaphases (6.7%) was higher than the one of metaphases (5.9%) and telophases (2.9%).

The lowest percentage of prophase cells was observed for regenerants obtained on MS supplemented with 2 mg IAA – 72.6%, but the highest percentage – 90.8% belongs to vitro-plants regenerated on BB_1 medium.

The percentage of metaphase cells was much lower compared with the prophases, ranging between 4.2 – for regenerants on BB_1 and 15.1 for regenerants on the medium A_2.

The percentage of cells in anaphase fluctuated in proximate limits (2.0 – 2.6) for 5 samples, excepting the control sample in which the anaphases represented 6,7% and the vitro-plants regenerated on medium A_2 for which the percentage of anaphases amounted to 5.3%.

For the cells in telophases, the percentage ranged from 2.4% - vitro-plants obtained on BA_1 and 6.8 –vitro-plants obtained on A_2. The control variant registered 2.9% cells in telophase.

The spectrum and frequency of aberrations identified varied with the type and concentration of growth regulators from nutritive mediums on which the vitro-plants regenerated.

The frequency of ana-telophases with chromosomal anomalies was generally reduced, but varied as follows: 8.54% at the control variant, 12.42

– N_2 variant, 13.33 – BN_1 variant, 16.42 –BB_1 variant, 16.6 – A_2 variant, 18.56 – KN_1 variant and 21.56 – BA_1 variant, (fig. 2).

The spectrum of chromosomal anomalies observed involved anaphases with: (simple and multiple) bridges, retarding chromosomes, fragments, delayed and expelled chromosomes and cells (prophases and interphases) with micronuclei, (photos 3-12).

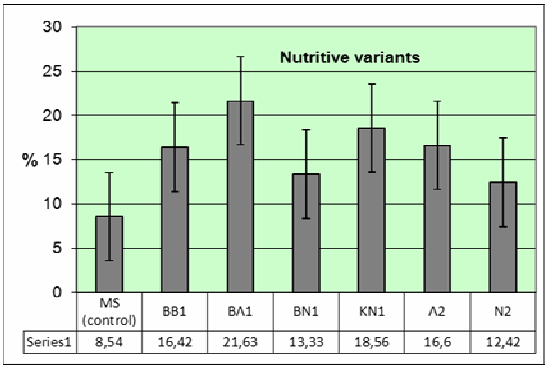

Fig. 2. The percentage of aberrant A-T cells observed at regenerants of *Veronica officinalis* L.

The highest frequency was recorded by A-T with bridges (67.9%), followed by A-T retarding chromosomes (50.0%), A-T with expelled chromosomes (26.1%), A-T with fragments (17.3%) and A-T with micronuclei (7.1%).

Along our investigations, we found ana-telophases with multiple (complex) aberrations, but with reduced frequency.

Among the chromosomal aberrations, the presence of micronuclei was identified only in 3 variants: MS, BN_1 and A_2. For the obtained vitro-plants on BN_1 and A_2 were observed ana-telophases with fragments.

In the frequency of aberrant ana-telophase cells, there is significant variation between the studied samples concerning the same type of chromosomal anomaly. Thus, the most numerous A-T with chromosomal bridges (fig. 3) were registered for BA_1 variant, but the lowest percentage of this type of aberration was calculated for N_2 variant.

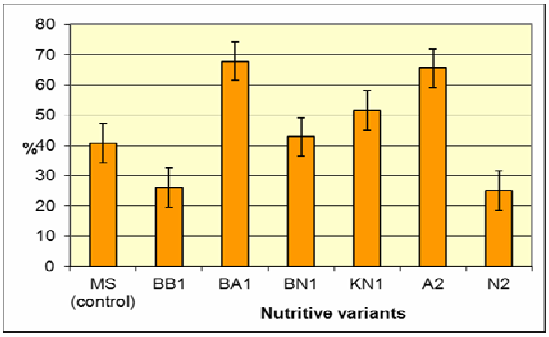

Fig.3. AT percentage with chromosomal bridges

The frequency of cells with retarding chromosomes (CR) varied between 50.0% - vitro-plants obtained on N_2 and 20,6% - vitro-plants obtained on A_2 (fig.4).

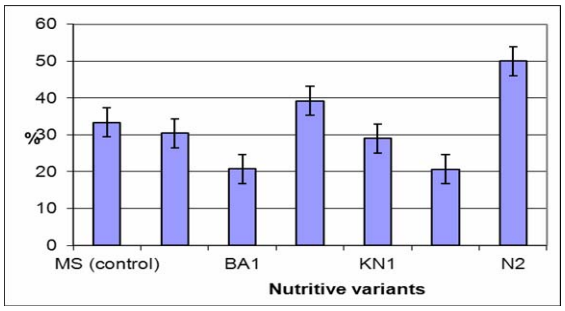

Fig. 4. The percentage of A-T cells with retarding chromosomes

The A-T with fragments varied depending on the hormonal formula of the nutritive medium, the highest percentage being registered for vitro-plants on BB_1 (17.3%), and the lowest for regenerants obtained on BA_1 (1.8%), (fig. 5).

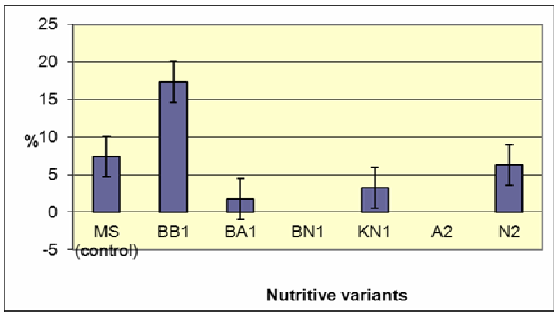

Fig. 5. The percentage of A-T cells with fragments

The highest percentage of A-T with expelled chromosomes was calculated for regenerants BB_1 – 26.1%, and the most reduced for vitro-plants obtained on A_2 – 9.1%, (fig.6).

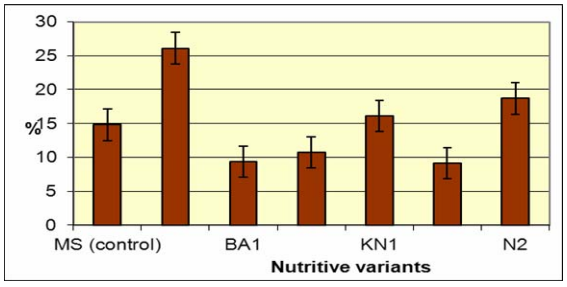

Fig. 6. The percentage A-T cells with expelled chromosomes

In the case of ana-telephasic cells with micronuclei for BN_1 variant there was calculated the

highest frequency – 7.1%, followed by A_2 variant where this type of aberration represented 4,5% and MS variant with an A-T frequency with micronuclei of 3.7%, (fig.7).

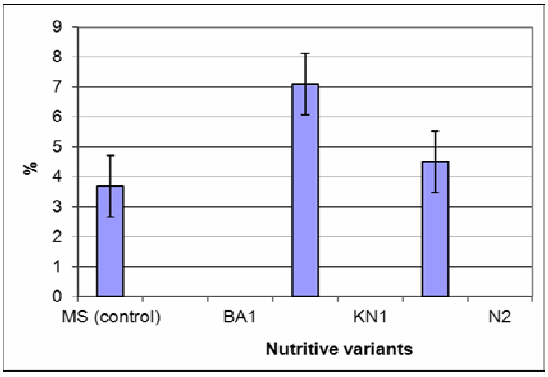

Fig.7. The percentage of cells with micronuclei

If analyzing the frequency of aberrant ana-telophase cells/type of vitro-plants analysed, we observe that the control variant (vitro-plants from MS) manifested the least disruptive elements, of which, the highest level was represented by A-T with chromosomal bridges (40.7%), and the lowest by A-T with fragments (3.7%).

For variant BB_1, A-T with retarding chromosomes recorded the highest frequency of all chromosomal aberrations (30.4%), followed by A-T with expelled chromosomes (26.1%), A-T with bridges (26.0%) and the lowest frequency in A-T with fragments (17.3%).

In the case of variant BA_1, the highest percentage was held A-T with chromosomal bridges (67.9%), the lowest A-T cells with fragments (1.8%).

For the vitro-plants regenerated on BN_1, KN_1 and A_2, the largest share is detained by A-T with bridges (42.8%, 51.6% respectively 65.5%). The most reduced frequency calculated for A-T with micronuclei (7.1% and 4.5%) for variants BN_1 respectively A_2. The most reduced frequency for variant KN_1 was calculated in A-T with fragments (3.2%).

Concerning the regenerants obtained on MS medium supplemented with 2 mg/l NAA, the best represented were the A-T with retarding chromosomes (50.0%), while the least numerous AT aberrant cells were found in cells with fragments (6.25%).

Chromosomal aberrations were identified in the metaphase and prophase of mitotic division, represented through fragments, expelled chromosomes or micronuclei, but their frequency was reduced.

CONCLUSIONS

- The cytogenetic observations on the radicular meristems of *Veronica officinalis* vitro-plants regenerated on 7 nutritive variants did not register malfunctions in the mitotic apparatus, that is, there were cells registered in all division phases.
- The cytogenetic studies outlined the fact that the mitotic index (MI) was high and its variation depended on the hormonal formula introduced in the nutritive medium, the highest MI being registered for vitro-plants coming from BA_1 nutritive variant (45,63), while the lowest was for vitro-plants BB_1 (30.7).
- The distribution of cells on diverse phases of mitotic division is, in general, the same, the highest frequency being recorded by prophases, followed by metaphases and telophases, while the lowest is for anaphases.
- In the case of regenerants obtained on MS basal medium without added phytohormones (control variant) the percentage of anaphases (6.7%) was higher than the one of metaphases (5.9%).
- We consider theta the frequency of cells with aberrations and the types of chromosomal aberrations registered in radicular meristems of speedwell vitro-plants were primarily determined by the genetic constitution of each explants and then by the hormonal formula of the nutritive mediums, on which they developed.
- The frequency of cell presenting chromosomal aberrations was relatively higher compared with the control variant and a relatively large spectrum of chromosomal anomalies identified, considering that the regeneration was produced through direct organogenesis from apexes and nodes of inoculated explants and the culture conditions were the same for all variants.
- In the chromosomal anomalies identified, the radicular meristems of speedwell , the highest frequency was recorded for A-T cells with bridges, followed by A-T with retarded chromosomes, A-T with expelled chromosomes and A-T with fragments. Their share varied on different nutritive mediums on which the vitro-plants were regenerated.
- In addition to ana-telophase cells with chromosomal aberrations, there were also identified interphases and prophase cells with micronuclei, but their frequency was reduced.

ABSTRACT

The cytogenetic studies conducted on radicular meristems of *Veronica officinalis* vitro-plants aim at indicating whether and to what extent the nutritive medium and the *in vitro* culture conditions influence the mitotic activity of cells. The investigation of the cytogenetic samples demonstrated the fact that the mitotic activity in radicular meristems is normal and there are no significant events. The mitotic index (MI) was calculated for the vitro-plants investigated and their

value varied according to the hormonal formula used in preparing the nutritive medium. The lowest MI (30.70) was calculated in the case of vitro-plants obtained on the MS medium supplemented with 1mg/l BAP and 0.5 mg/l IBA, and the highest MI value IM (45.63) was calculated for regenerants obtained on BA_1 variant (1mg/l BAP and 0.1 mg/l IAA).

Concerning the distribution of mitotic division phases, cells were observed to be predominant in prophases, followed by metaphases, telophases and anaphases at the majority of the radicular meristems of the vitro-plants studied.

The frequency and spectrum of chromosomal aberrations also varied according to the nutritive variants on which the vitro-plants were regenerated. The spectrum of chromosomal anomalies involved anaphases with: (simple and multiple) bridges, retarding chromosomes, fragments, delayed and expelled chromosomes and cells (prophases and interphases) with micronuclei.

In the case of the *in vitro* culture technology applied for cloning the species *Veronica officinalis*, the cell division has not been disturbed, the values of the mitotic index calculated for the regenerated vitro-plants on the mediums with phytohormones being proximate to the ones of MS medium regenerants without phytohormone supplements. The number of cells identified with chromosomal aberrations was relatively close to the one of control vitro-plants.

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Photo 1. Vitro-plants (BB1 nutritiv varint)

Photo 2. Roots of vitro-plants on A2 medium

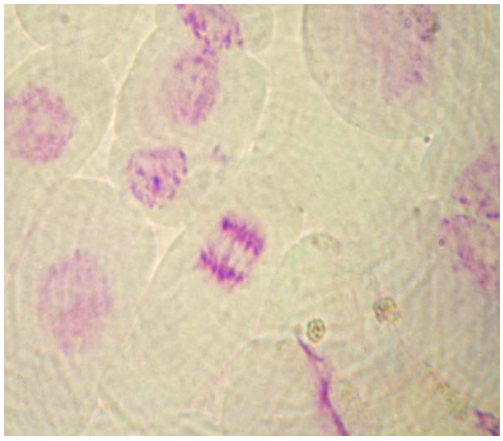

Photo 3. Anaphase with bridges (BA_1)

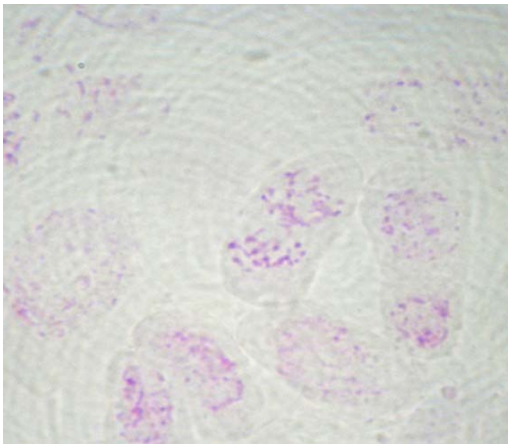

Photo 4. Disorganized anaphase (BB_1)

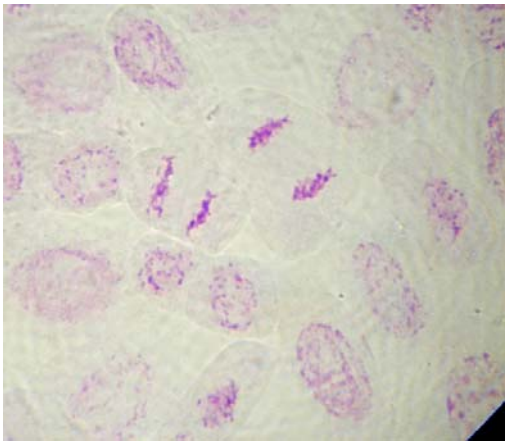

Photo 5. Ana-telophase with fragment and expelled chromosoms (BA_1)

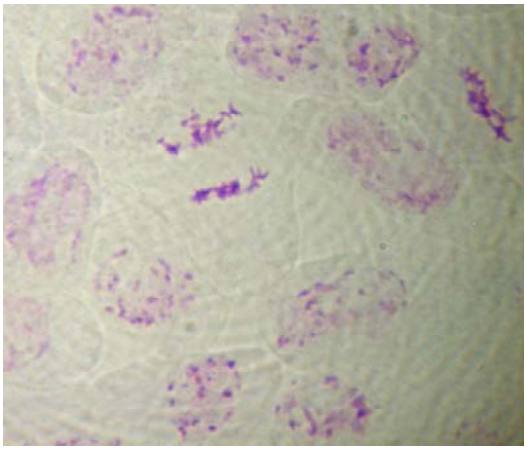

Photo 6. A-T with expelled chromosom (KN_1)

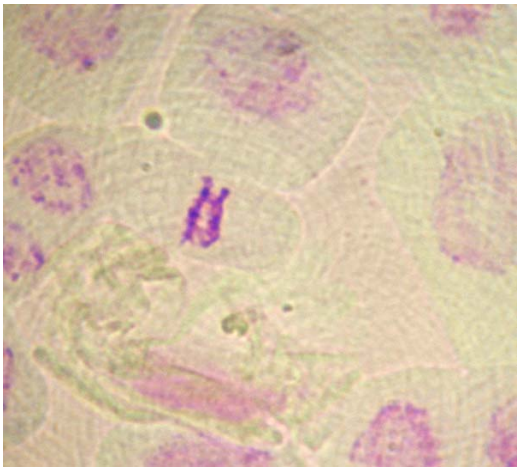

Photo 7. Anaphase with bridges

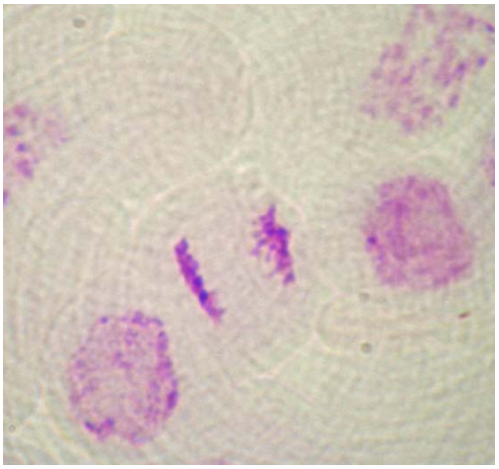

Photo 8. Telophase with delayed chromosomes (A_2)

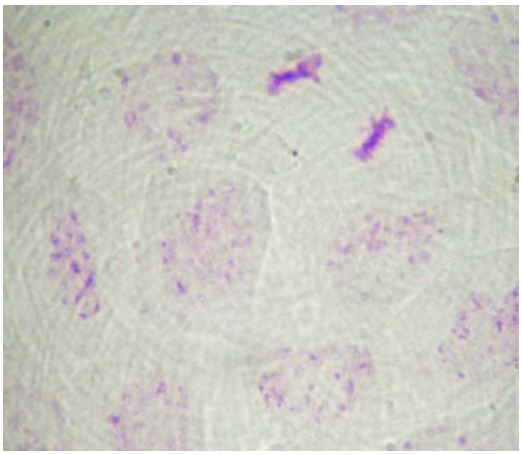

Photo 9. Telophase with delayed chromosome (BB_1)

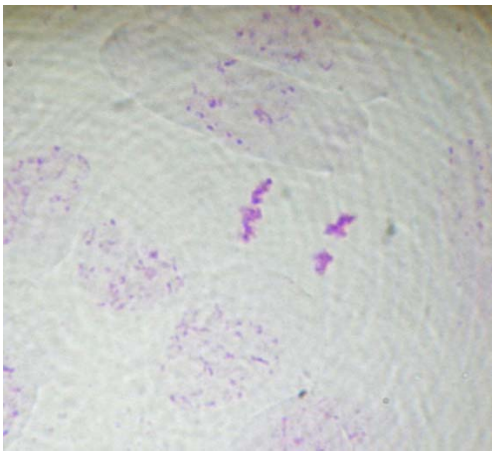

Photo 10. Telophase with gap (BN_1)

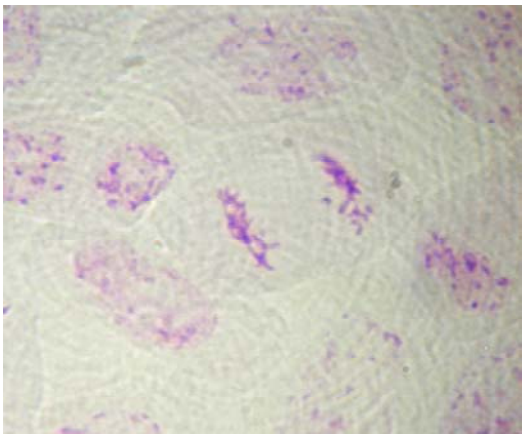

Photo 11. Telophase with fragment and expelled chromosoms (MS)

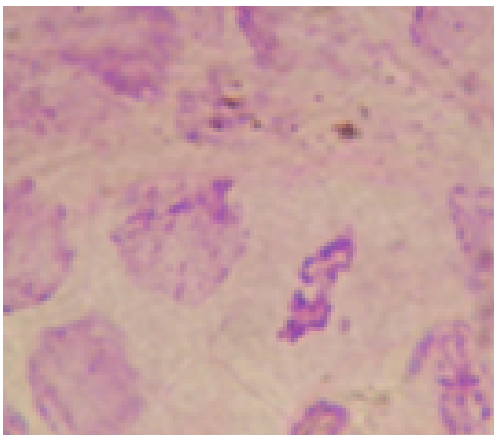

Photo 12. Prophase with micronucleu(A_2)