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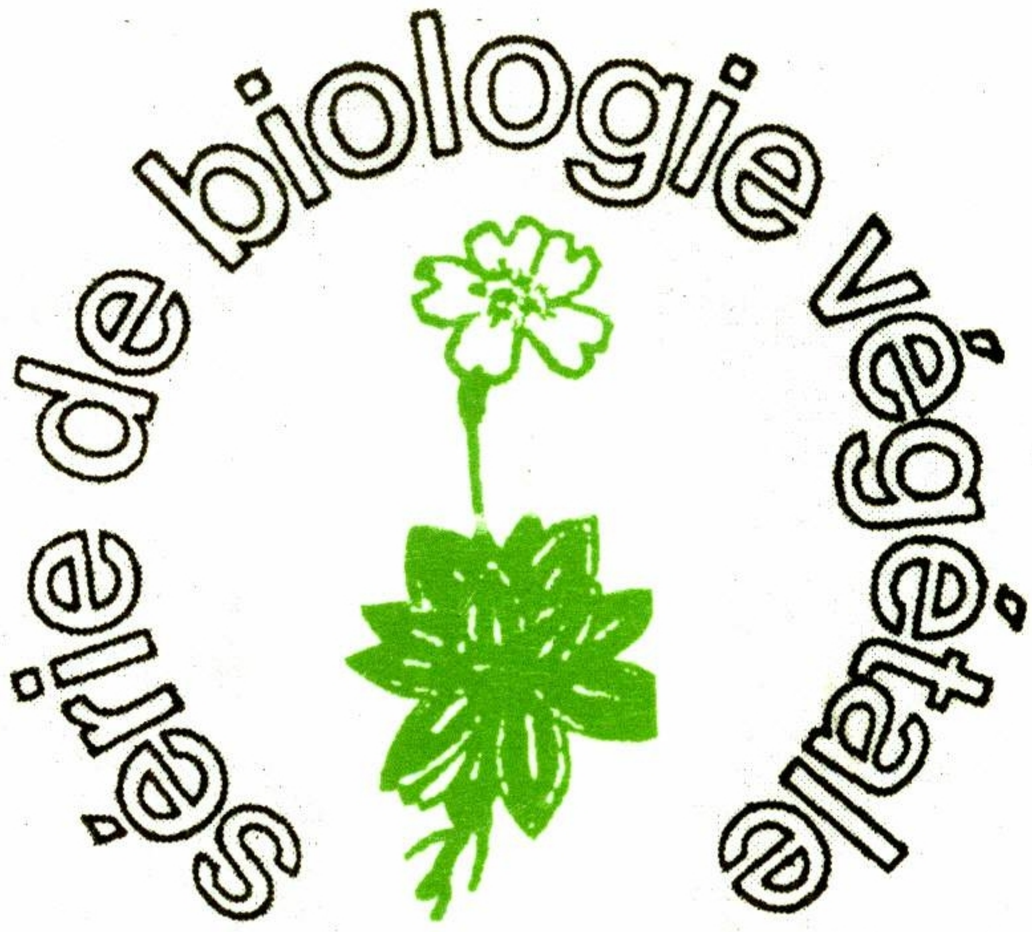


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## SÉRIE DE BIOLOGIE VÉGÉTALE

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# ELECTRONOMICROSCOPICAL EVIDENCE OF SPECIFIC INTERACTIONS BETWEEN WILD STRAINS OF *AGROBACTERIUM TUMEFACIENS* L. AND HOST PLANT CELLS

AURELIA BREZEANU*, CĂLINA PETRUȚA CORNEA**, ION VĂTAFU**

The interactions between virulent wild strains of *Agrobacterium tumefaciens* L. cells or spheroplasts and *Nicotiana tabacum* L. cells (from suspension culture), protoplasts and different cellular formations (cell wall, plasma membrane and chloroplast envelope) were analyzed at the electronomicroscopical level. The attachment of the bacteria to the surface of the cell was mediated by the bacterial projections, which seems to be exopolysaccharides like cellulosic fibres. The fibrils produced by virulent bacteria are also involved in the attachment to the protoplast plasmalemma. Particular interactions between bacteria cells or spheroplasts respectively and the envelope of isolated chloroplast are discussed.

*Key words: A. tumefaciens*, *N. tabacum*, cells, protoplasts, spheroplasts, cellular interactions, electronomicroscopy.

## INTRODUCTION

*Agrobacterium tumefaciens* is a soil pathogen that genetically transforms host plants, causing crown gall tumors, an agronomical important disease that affects most dicotyledonous plants. The *Agrobacterium*-plant cell interaction is the only known natural example of DNA transport between kingdoms (12). Cell-cell contact is required for the onset of many intracellular interactions, including host-pathogen recognition (1, 3, 4, 5). *Agrobacterium* carries three genetic components required for plant cell transformation. Two components, the T-DNA and the virulence region (*vir*), are located on the large Ti plasmid. The third bacterial component, needed for the T-DNA transfer process, resides in the *Agrobacterium* chromosome. Three chromosomal virulence loci encoded products involved in the binding of *Agrobacterium* to the plant cells during the infection process (9, 10).

This work concerns the study of the interactions between *Agrobacterium tumefaciens* L. cells and spheroplasts and *Nicotiana tabacum* L. cv. xanti cells, protoplast and different cellular components (cell wall, plasma membrane and chloroplast envelope) at the electronomicroscopical level.

## MATERIAL AND METHODS

1. *Plant material – Nicotiana tabacum L. cv. xanti cells from cell suspensions and protoplasts were used as biological material*

a) Plant suspensions of *Nicotiana tabacum* L. were obtained from callus culture which was initiated by petiole explants on a variant of Murashige-Skoog (7) basal medium containing NAA 2.0 mg/l and kinetin 0.2 mg/l. 1g of fresh callus per 100 ml nutritive liquid medium with the same composition was used as inoculum source for cell suspension culture initiation.

b) As a protoplast source, foliar mesophyll from *Nicotiana tabacum* L. *cv. Xanthi* plants obtained by aseptic germination of seeds was used. Protoplast isolation was achieved according to the method described by Brezeanu *et al.* (2) with slight modification. Mesophyll fragments were incubated in an enzyme solution without shaking for 16 hours at 24°C, in the dark. The enzyme solution contained 0.5% cellulizin (Serva) and 0.5% macerozyme (Serva) in 0.7M mannitol; prior to use it was sterilized by filtration. After the incubation, the protoplasts were isolated and purified by filtration through nylon sieve (62–70 μm), followed by three successive centrifugations (5 minutes at 100 g). The pellet was resuspended in osmotic stabilizing solution of 0.7M mannitol, pH 5.8, followed by another centrifugation for 8 min at 100 g on a 23% sucrose gradient. Intact protoplasts which formed a light-green layer at the mannitol-sucrose interface were recovered and subsequently used in our experiments.

2. *Bacterial strains*

A wild strain of *Agrobacterium tumefaciens* A6 (a tumorigenic strain) was used in our experiments. The bacteria were maintained on YMA medium (yeast extract-mannitol-agar medium) and cultivated for 48 hours in Luria Bertani broth before use.

3. *Spheroplast isolation*

Bacterial cells were cultivated in TYN medium and to convert them to spheroplasts we used penicillin 6 mg/ml, for 6 hours as described in a previous paper (4), which in a modified protocol was described by Olass (1983).

4. *Co-cultivation*

The interactions between bacteria (both intact cells and spheroplasts) and plant cells (cells from suspension and protoplasts) were observed after co-cultivation for 1 h, 4 h and 24h.

5. *Electronomicroscopy*

Samples were processed by the standard method with some modifications: fixation in 3% glutaraldehyde in phosphate buffer (0.2M) for 18 h at 4°C, postfixed in 2% $OsO_4$ for 2 h at room temperature and embedded in Epon 812 resin. The ultrathin sections, obtained with Tesla ultramicrotome, were stained by Reynolds's method and visualized under TESLA BS500 electron microscope. To study the interactions between plant protoplasts and bacteria, after glutaraldehyde fixation samples were embedded in 2% agar at 37°C for easier manipulation.

**Plate I** – Successive stages of adhesion between bacterial cells (*A. tumefaciens*) and plant cell (*N. tabacum*):

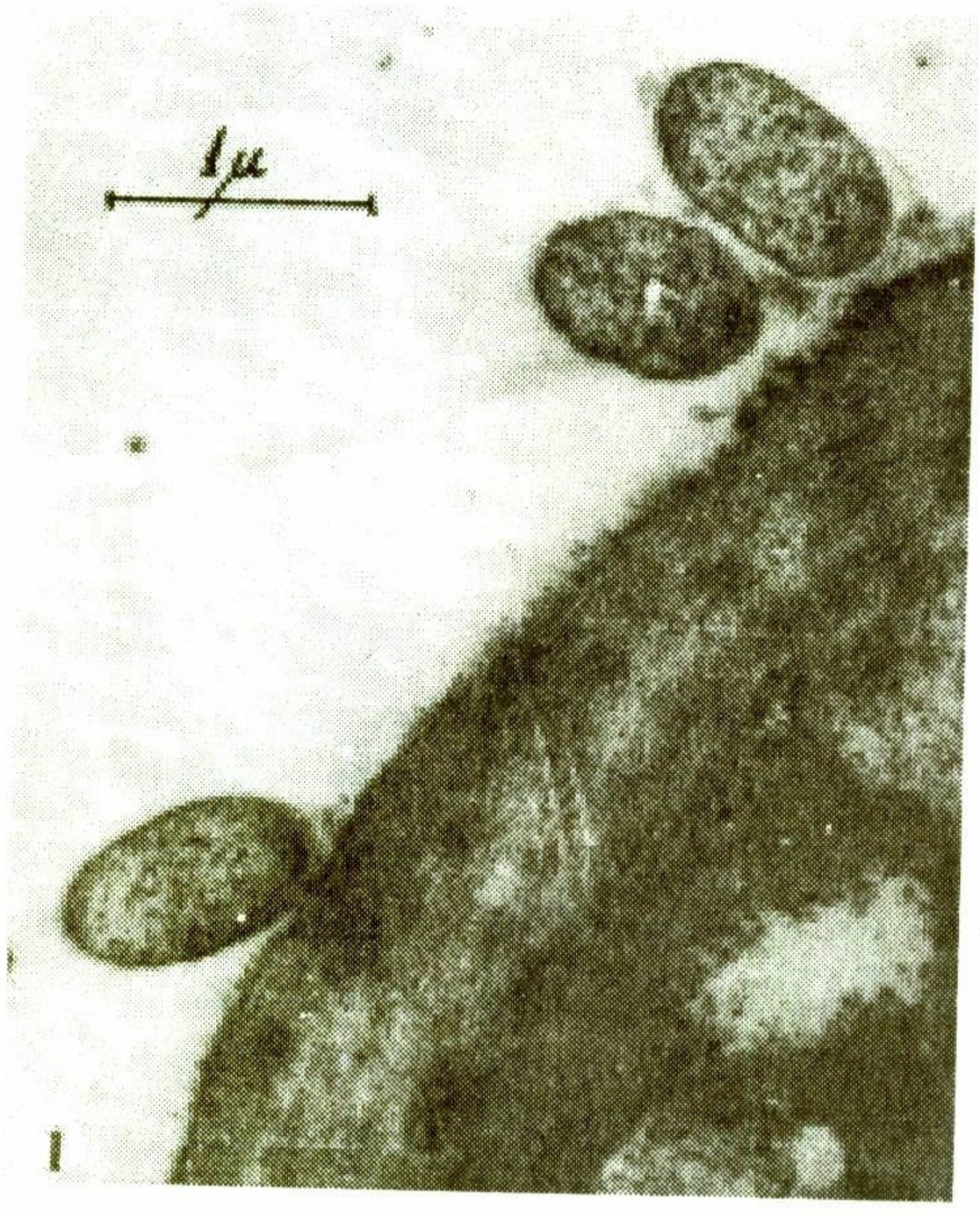


Fig. 1 – First stage of adhesion.

Fig. 2 – The bacterial projections (cellulosic fibrils like) located in the adhesion area only (see arrow).

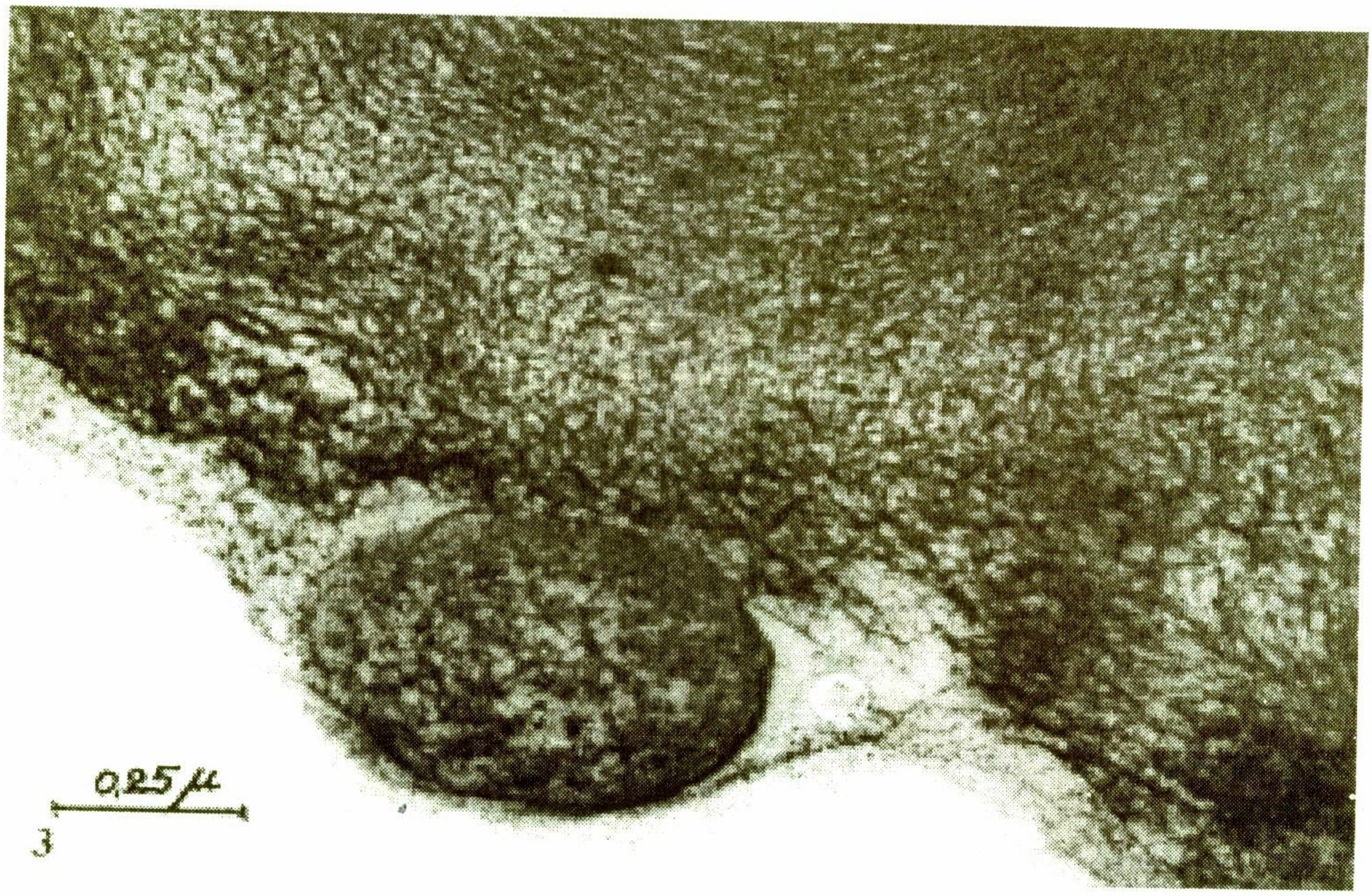


Fig. 3 – The bacterial cell completely surrounded by "cellulosic fibrils like" derived both from bacterial and plant cells.

**Plate II**

Interactions between:

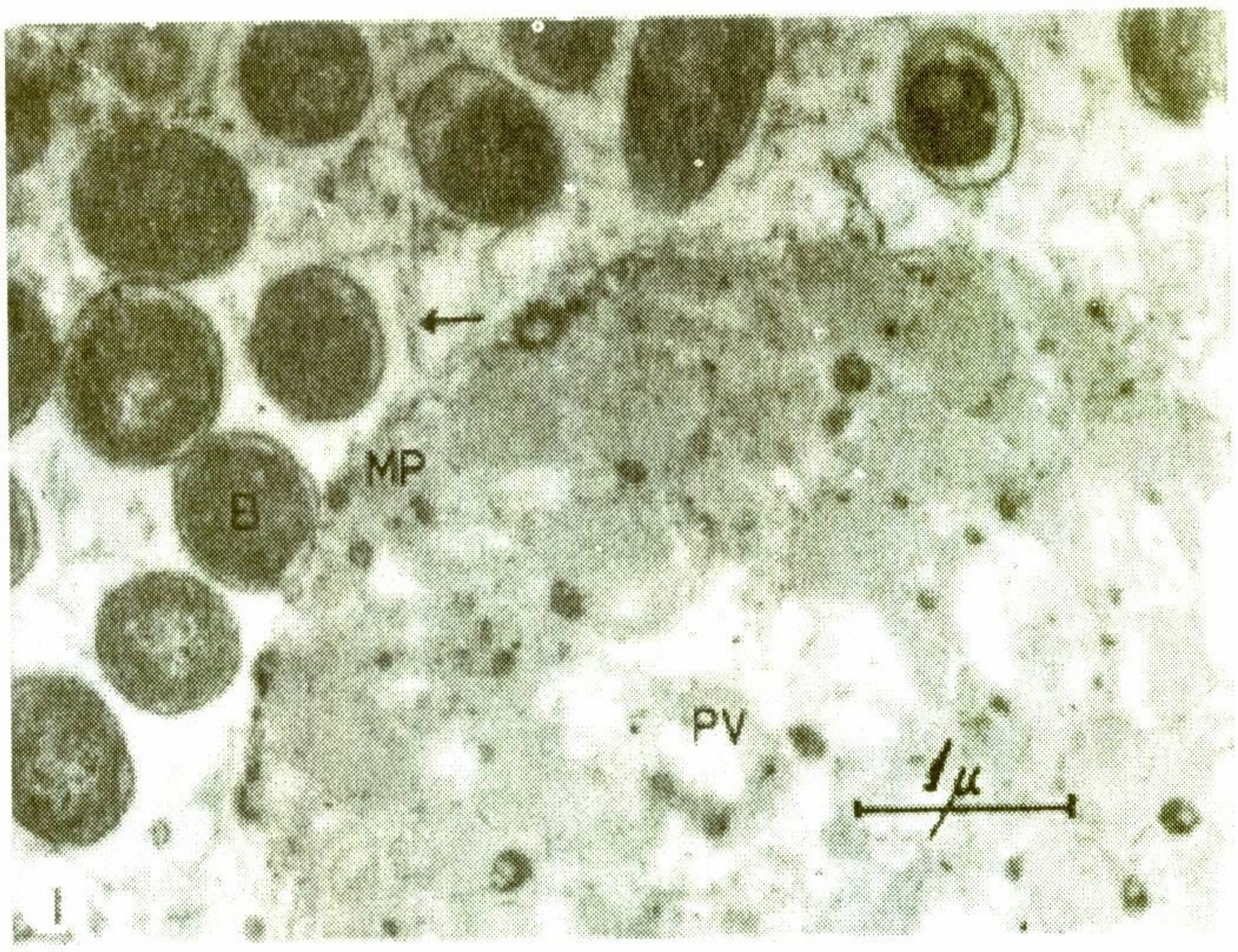


Fig. 1 – Virulent bacterial cells and *N. tabacum* protoplast.

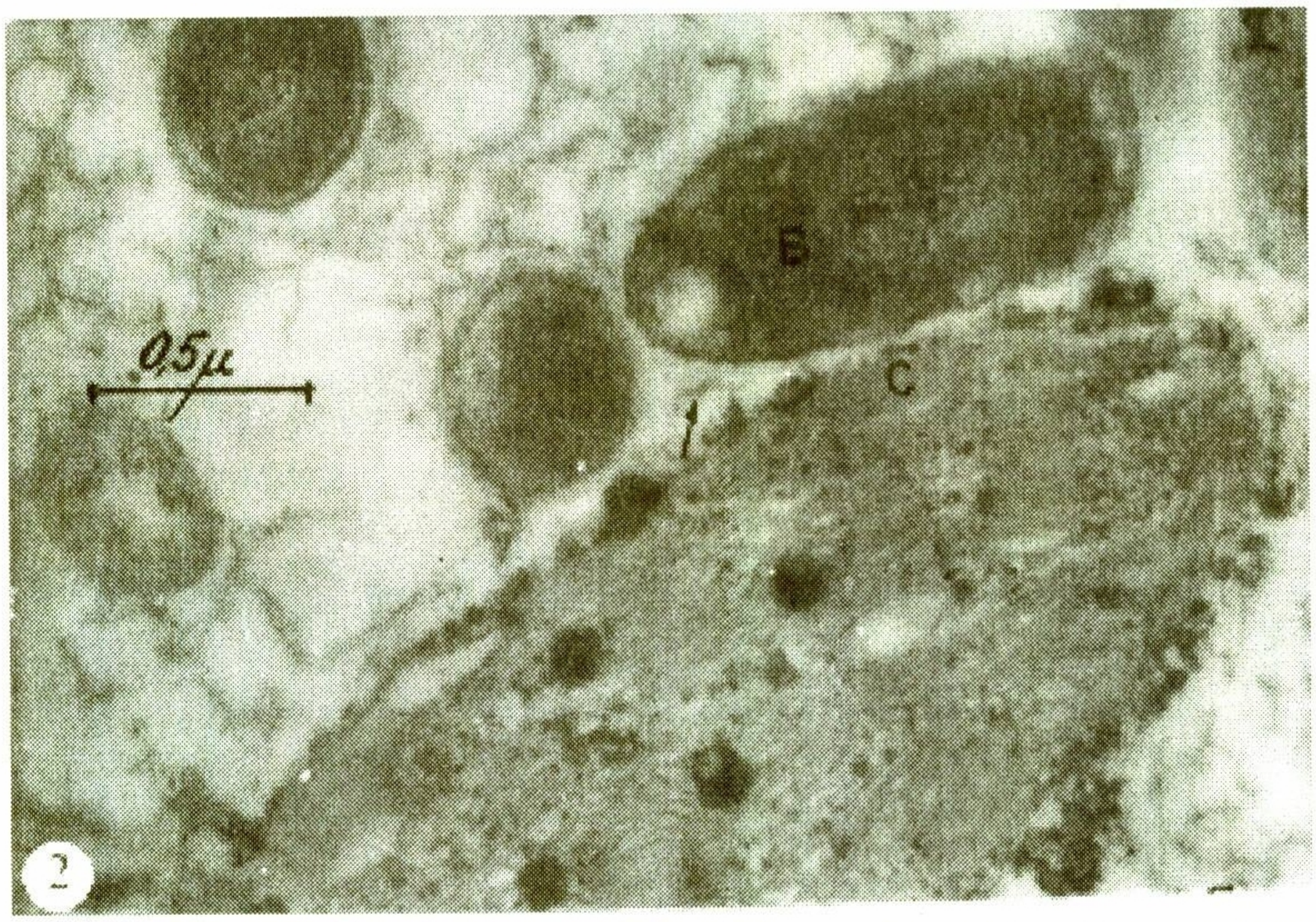


Fig. 2 – Bacterial cell and the external membrane of the chloroplast envelope.

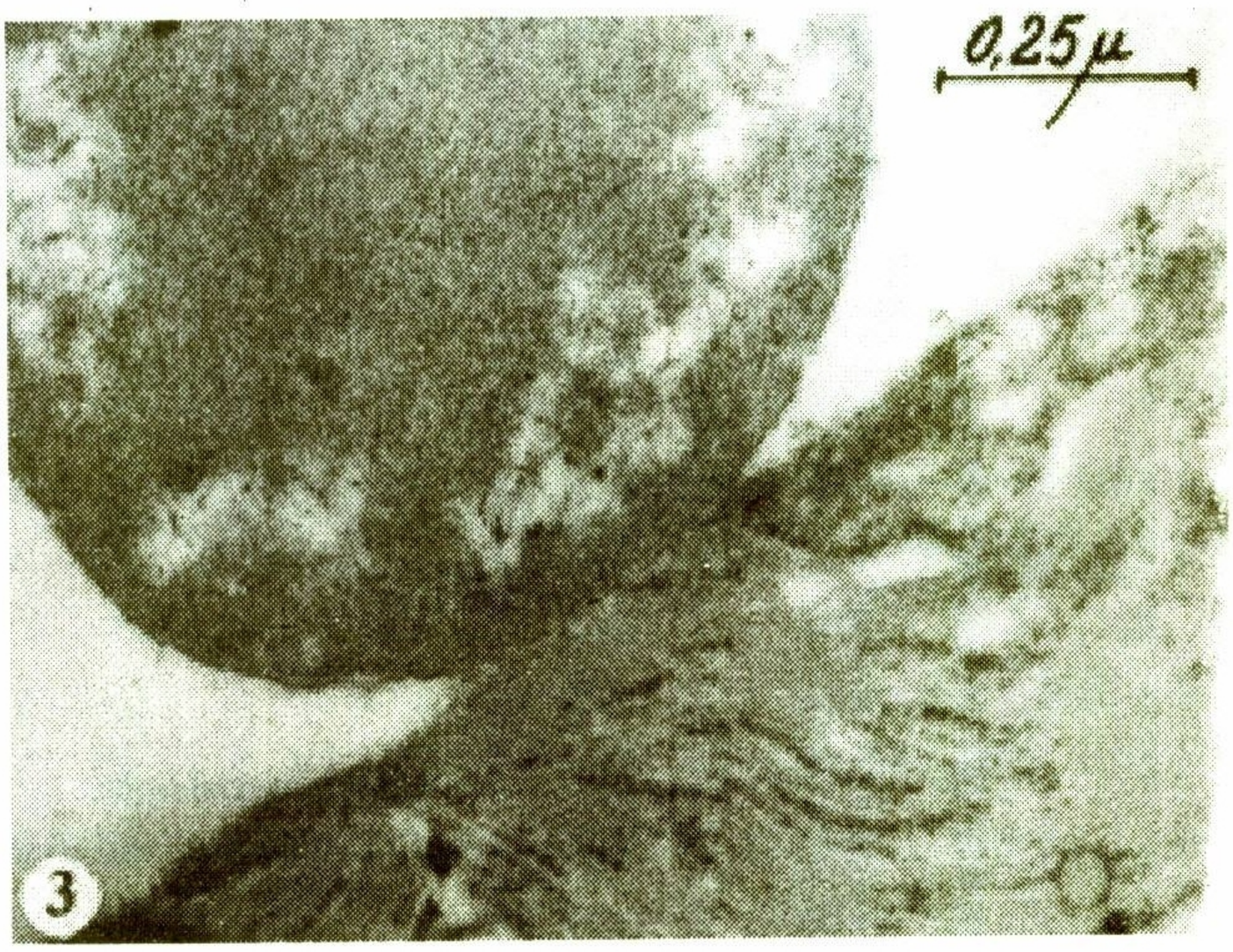


Fig. 3 – Bacterial spheroplasts and tobacco protoplast.

## RESULTS AND DISCUSSIONS

Initial steps of the interaction of plant and *Agrobacterium* cells are: chemical recognition of wounded plant cells by *Agrobacterium*, followed by chemotaxis towards these cells; attachment to the plant cell wall and induction of *vir* functions. The first step of successful genetic colonization by *Agrobacterium* involves the recognition of those plant cells, which are competent for transformation. "*In vivo*" the infection requires wounding of the plant tissues which induces a series of metabolic pathways and the subsequent accumulation of metabolites including sugars, amino acids and phenols (10). These compounds are effective chemoattractants for agrobacteria (9). When plant suspension or protoplasts were used for "*in vitro*" co-cultivation with bacterial cells, it is possible that some plant cells accumulate and release the compounds necessary to initiate the infection process.

Once attracted, bacteria attach to the cell walls of the competent plant cells. The molecular basis for attachment is not clear but some microscopic evidence could help to understand the aspects of this step.

Many authors have reported those chromosomal virulence loci; *chvA, chvB* and *pscA* may provide the compounds, which allow *Agrobacterium* to form an effective contact with recipient plant cells. When *Agrobacterium* and *N.tabacum* cells (in suspension) were co-cultivated for one hour, the bacterial cells started to be attached to the cell wall (Plate I, Fig.1).

After 4 h of co-cultivation, bacterial projections were observed to mediate the attachment to the surface of the cell wall (Plate I, Fig.2). Mathysse (6) analyzed the biochemical nature of these projections: it seems to be involved exopolysaccharides (EPS) like cellulosic fibers. As it can be seen in Fig. 2, the fibrils are present only in the restricted area, where bacteria are located. In some situations, the bacterial cells are surrounded by a material, possibly derived both from bacterial cells and plant cells (Plate I, Fig.3).

It was shown (11) that mutants of chromosomal *pscA* locus are deficient in the production of polysaccharides and lack the ability of attachment. Moreover, similar interactions were reported for actinorrhizal species, but in this case it was demonstrated that the host cells produce EPS as response to rhizosphere bacteria (1). It seems that the fibrils produced by virulent bacteria are also involved in the attachment to cytoplasmic membrane of the protoplasts (Plate II, Fig.1).

It is possible that the fibrillar material synthesized by bacteria mediate a tight and irreversible association between agrobacteria and the host cell (to the cell wall or cytoplasmic membrane). Similar interactions were observed between intact bacteria and chloroplast membrane (Plate II, Fig.2). This evidence could be explained by the presence of specific plant cells surface protein and carbohydrates, which function as specific receptors for bacteria. However, the identity of these molecules remains to be determined (6, 12). Interesting aspects were observed

when we examined the interactions between *Agrobacterium* spheroplasts and *N. tabacum* protoplasts. Tight contacts between the adjacent membranes: bacterial, protoplasts or chloroplast membrane (Plate II, Fig.3), similar to that observed in cellular fusion (protoplast fusion) (8). No bacterial projections (cellulosic filaments) were observed in these situations.

It is known that protoplasts present on their surface short chains of polysaccharides, also AGP that may function as binding sites for bacterial cell (13). The chloroplast envelope contains over 70% proteins, part of them might function as receptors of bacterial cells or spheroplasts. These data suggest that the interactions between bacteria and plant cells involved more complicated mechanisms of recognition. It is possible that the presence of cell wall and wounding effect is not obligatory for the attachment of bacteria, the step being not specific. For that reason, the attachment could be realized at the level of different types of cellular membranes. It is possible that another factors like enzyme solution used for protoplasts or spheroplasts isolation, composition of the nutritive medium in carbohydrates and bivalent ions ($Ca^{2+}$, $Mg^{2+}$, etc) to represent activators for the adhesion process and might be real the hypothesis of Mathysse by which the induction of bacteria cellulose synthesis might be stimulated only in the presence of some plant extracts (6).

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# SEX CHROMOSOMES IN SOME DIOECIOUS SPECIES OF *RUMEX* GENERA OF THE ROMANIAN FLORA

NICOLETA CONSTANTIN*, ANDREEA CUNIȚĂ*, GAVRIL NEGREAN**, VERONICA STOIAN*, ANAMARIA LOGOJAN*

Among various plant sex systems the dioecious condition is very rare; newer data reveal its association with wind and water pollination. Our work deals with sex chromosome study in two species of *Rumex* genus of the Romanian flora. Taxonomic and ecological data are presented and chromosomal constitutions are discussed.

*Key words:* dioecious species, sex chromosomes, *Rumex* genera.

## INTRODUCTION

Among various sex systems, in taxons with morphological complex, highly specialized, big flowers such as those pollinated by birds, bats, bees, etc., the dioecious condition is very rare; newer data reveal its association with wind and water pollination. In comparison with monoic subspecies, the dioecious plants present a higher degree of allelic diversity and heterozygosity; consequently, their selective advantage is the avoidance of the depression due to autofertilization and inbreeding. It is generally accepted that sex is also a consequence of the requirement of suppressing the negative effect of recombination and mutation (3). Another important aspect is the smallness of families manifesting this sort of sexuality; they include dioecious genera usually related with monoecious taxons. Similarly, dioecious species (which are nevertheless a minority) belong to genera that include monoecious and also hermaphrodite species (15). This fact indicates that, in some circumstances, sex separation might represent an immediate advantage; on the other hand, the small number of dioecious taxons shows that this advantage would rarely be important enough to offer evolutive diversification possibilities (6). This is strikingly opposite to the wide incidence of separated sexes in animal kingdom (7). Given all these facts, dioecious plants represent very good instruments to study genetical basis of sex determination and offer the possibility to study the evolution monoecious ↔ dioecious ↔ hermaphrodite condition.

In the Romanian flora, there are 109 dioecious taxons of spontaneous or subspontaneous tracheophytes representing 2.78% of their total number; besides, in Table 1 are included other forms of sexuality. The best-represented family is *Salicaceae,* with 35 taxons (all of the European species are dioecious). It is followed by *Cariophyllaceae* family, with 13 taxons, all of them belonging to *Silene* genus, (especially to *Otites* subgenus), and afterwards, by *Asteraceae* family (9 taxons) and *Polygonaceae* (8 taxons).

The biological analysis of dioecious species shows the high incidence of phanerophytes (49 taxons), most of them from *Salicaceae* family (allogamous, anemophilous). Hemicryptophytes are represented by 24 taxons. Almost all of the 15 geophytes belong to *Asparagus* and *Petasites* genera. 8 species are annual weeds. As for their origin, most of the dioecious taxons are Eurasiatic. 11 species are adventive (almost all, American native weeds). 15 taxons are endemic for Europe, and 34 species are rare, vulnerable and/or endangered (1,2,9). From the phytosociologic point of view (taking into account only large vegetal formations), 57 taxons grow in forest zones, and 23 on meadows. Many species are ruderal, all adventive plants included.

## MATERIALS AND METHODS

The samples were collected from five plant populations, as follows:

– *Rumex acetosa* inflorescences, from Poiana Ţapului, Jud. Prahova (25°32'20''N, 45°23'32''E and altitude 860m), from Sinaia – Cumpătul (25°32'40''N, 45°22'30''E and altitude 830m) and Sălătrucu, Jud. Argeş (24°30'40''N, 45°18'50''E and altitude 700 m) in June 1998; seeds – from Secu – Valea Topologului, jud. Argeş (24°31'00''N, 45°20'20''E and altitude 600m) and Sălătrucu – Argeş (same location as above) in July 1998;

– *Rumex alpestris* inflorescences and seeds, from Sinaia – Poiana Stânii, jud. Prahova (25°31'25''N, 45°22'11''E and altitude 1300m) in June and July 1998, respectively.

In order to evidence meiotic and mitotic chromosomes, we used Feulgen coloration.

For C-banding, we used root tips hydrolyzed with 1N HCl (14 min, 60°C) and squashed in a 45% acetic acid solution. The slides selection was made in phase contrast microscopy. After removing the coverslip by dry ice method, the slides were dehydrated in absolute ethyl alcohol and dried at room temperature. The next day, the slides were treated with HCl (0,2 N, 60°C, 1 min) and then immersed in saturated $Ba(OH)_2$ solution (5 min, 60°C). After thorough washing, the slides were incubated in 2xSSC (60°C, 1 hour) and colored with 2% Giemsa in phosphate buffer; coloration time varied depending on species. Slides were analyzed with an Amplival microscope and photographed with an Exacta-Varex camera.

## RESULTS AND DISCUSSIONS

*Rumex acetosa* L. ssp. *acetosa* is a perennial dioecious plant with a large distribution; that denotes ecological tolerance and the fact that various ecological conditions do not affect the two sexes fitness (sex ratio is 1:1) (4). The plants are

about 80 cm high. Basal leaves are 2–6 times as long as wide, with the basal lobes moderately acute and ochreae are fimbriate. The branches of the inflorescence are single, directed upwards, branching only in the lower part of the inflorescence. The inner fruit valves are 3–3.5 mm long and seeds are dark brown, shiny. It flowers since May to June. The species is common throughout the lowlands of Europe, especially in North and Central Europe, in Subarctic and Central Asia, and in North America. The taxon grows on meadows, along field roads, and in wayside ditches (10,13).

*Table 1*

Numerical distribution of dioecious-like sex forms in Romanian flora

| Sex forms | Fam. | Genera | Species and subspecies |
|---|---|---|---|
| Dioecious | 27 | 38 | 109 |
| Dioecious or polygamous | 1 | 1 | 1 |
| Dioecious or hermaphrodite | 1 | 1 | 1 |
| Functional dioecious | 1 | 1 | 1 |
| Incompletely dioecious | 1 | 1 | 1 |
| Monoecious or dioecious | 2 | 2 | 2 |
| Polygamous or dioecious | 1 | 2 | 2 |
| Polygamous-dioecious | 1 | 1 | 1 |
| TOTAL | 35 | 47 | 118 |

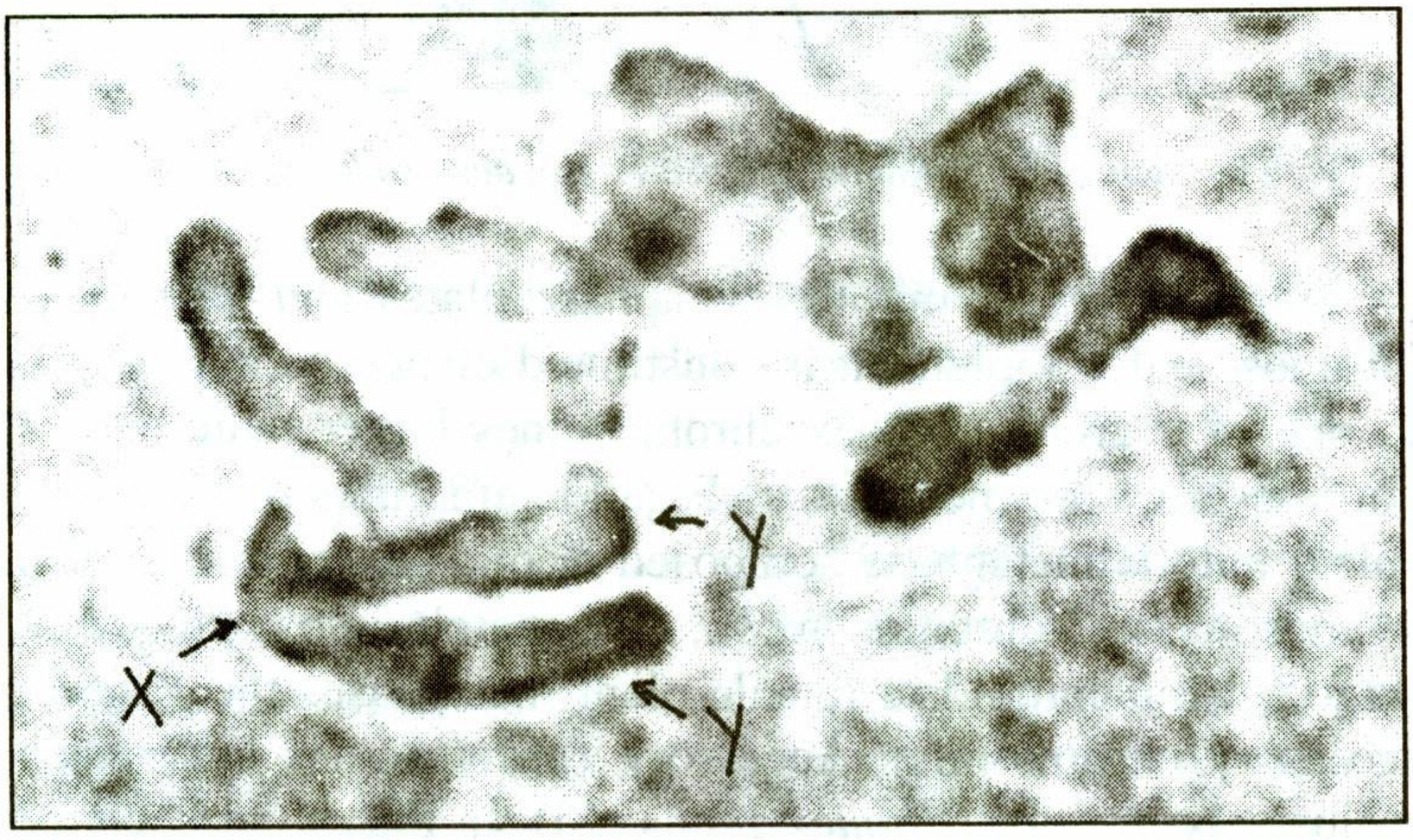


Fig. 1 – Male plant of *Rumex acetosa* L. ssp. *acetosa* – meiotic metaphase I.

The diploid number of chromosomes is 14 (12+XX) for female plants, and 15 (12+$XY_1Y_2$) for male plants, respectively (8). Świetlińska (11) cites four major autosomal types: *i*-group (terminal centromere and various length), *t*-group (satellited chromosomes), *v*-group (almost median centromere), *j*-group (submedian centromere, larger than *v* type). X chromosomes are the longest of the

entire complement and have a median centromere; Y chromosomes are shorter than X and with various positions of centromere. In metaphase I of pollen mother cell meiosis, the autosomes form six bivalents, and the sex chromosomes form one trivalent YXY (5). All autosomal bivalents and the tripartite sex chromosomes are uniformly stained (Fig. 1).

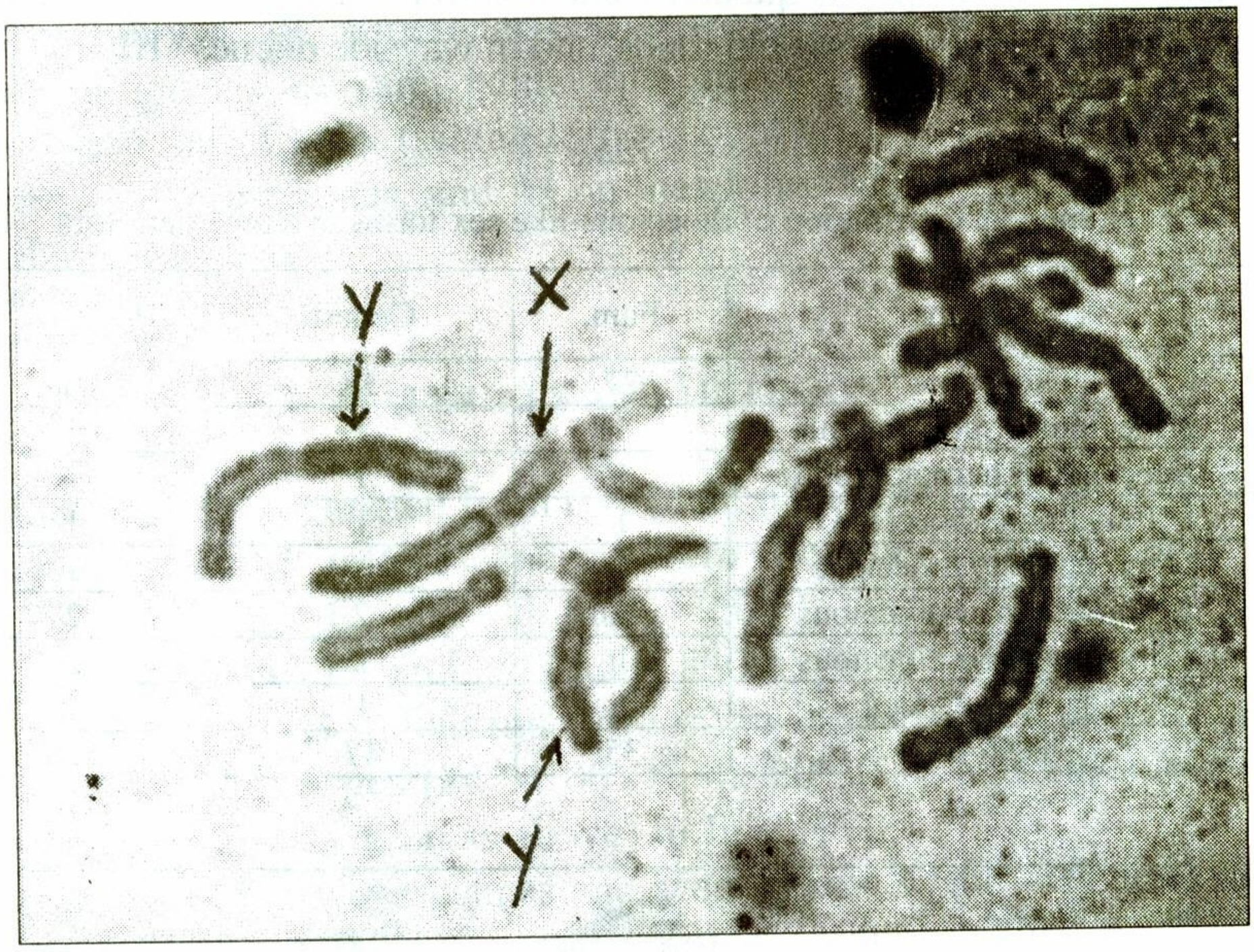


Fig. 2a – Male plant of *Rumex acetosa* L. ssp. *acetosa* – mitotic metaphase.

Figs. 2 and 3 present mitotic metaphase plates and karyotypes from male plants. Chromosomal complement is constituted of two pairs of *j* group, three of *i* group and one of *v* group and sex chromosomes larger than autosomes; in both cases Y chromosomes are metacentric. Fig. 4 exemplifies these autosomal types in an incomplete mitotic metaphase composed of one pair of chromosomes *t*, 2 pairs of *j*, 1 chromosome *v*, 3 chromosomes *i*, and one chromosome X and two Y (12). Also, in Fig. 5, we identified in female plant metaphases the X chromosome pair and an accessory chromosome. Accessory chromosomes are present in *acetosa* species exclusively, inside of *Rumex* genus (13). In Fig. 5 the X chromosomes pair is indicated in female plant metaphases.

As far as the C pattern is concerned, Y chromosomes are uniformly heterochromatic and present only a small euchromatic region at one of the telomeres (Fig. 6) that represents the pseudoautosomal pairing region. Autosomes present heterochromatic telomeric bands on one arm and interstitial bands. X chromosomes are similar to the autosomes and have only interstitial bands (Fig. 6,7).

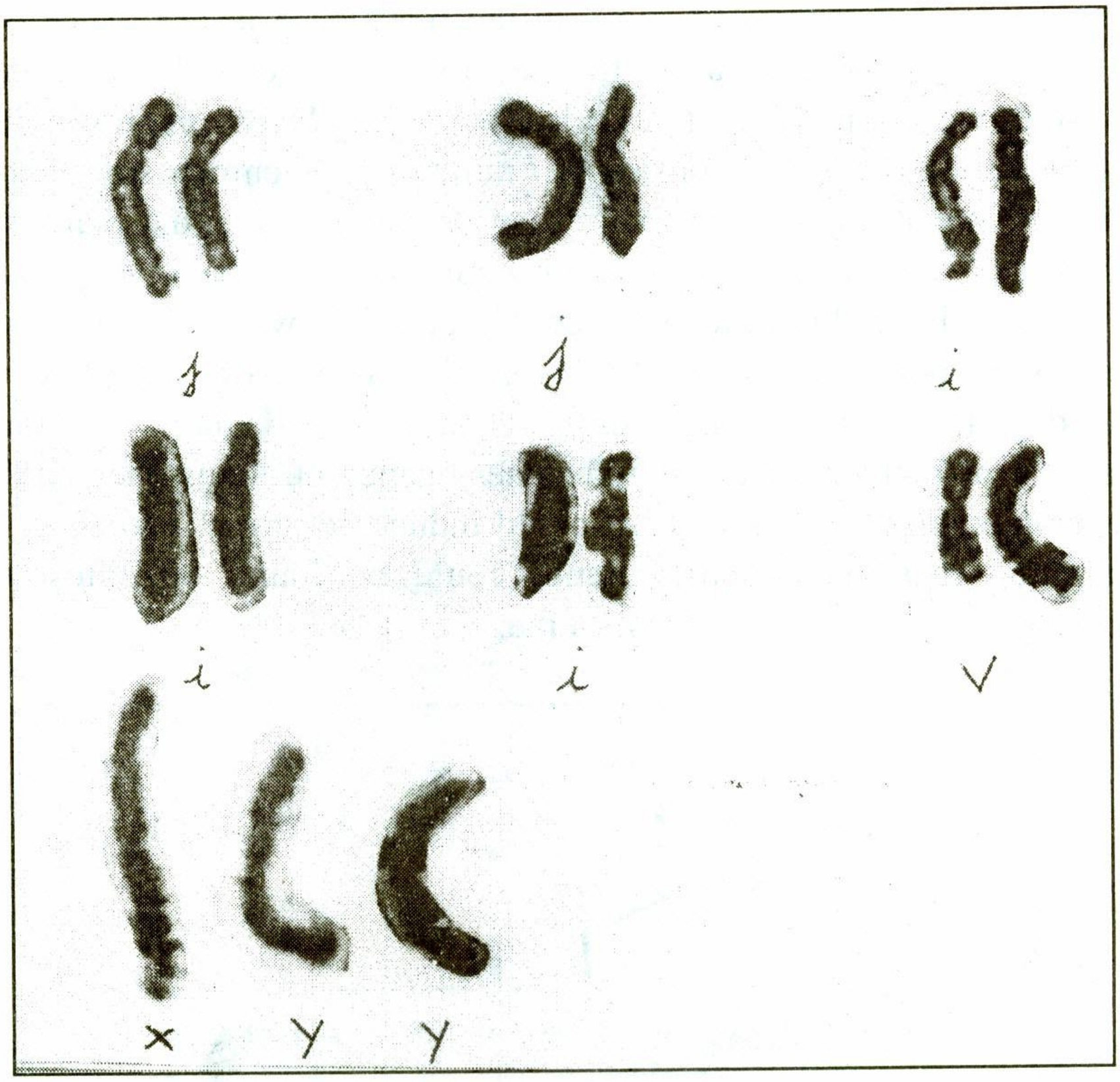


Fig. 2b – Male plant of *Rumex acetosa* L. ssp. *acetosa* – karyotype.

*Rumex alpestris* Jacq. s.l. is also a perennial dioecious species. Plants are up to 120 cm high. Basal leaves are about twice as long as wide, ovate with basal lobes usually obtuse. Ochreae are entire or slightly laciniate at top. The branches of inflorescence are simple. The fruit valves are 3.3–4.5 mm long and wide, usually with a yellowish-gray tint. Seeds are usually larger than those of *R. acetosa* ssp. *acetosa*. Flowering time lasts from May till August. It grows in forests and on wet meadows in the mountains. *R. alpestris* occurs in the mountains of Europe, Central Asia, North Africa, and Alaska. It also grows in the Siberian tundra (10, 13).

This species has the same chromosomal constitution as *R. acetosa* ssp. *acetosa* (12+XX for female plants, 12+XYY for male plants, respectively). Likewise, there are four chromosomal types *i, j, t, v*. Chromosomal constitution variability is high enough; different population constitutions (i.e. 12*i*, 9*i*+*j*+*t*, 11*i*+*t*, 11*i*+*j*) are mentioned in the literature (16). X chromosomes are the largest of the complement with median centromere. The second in range is Y chromosome (almost median centromere). The karyotype in Fig. 8 is constituted of 12 type *i*

autosomes and 3 gonosomes (X+2Y). In the metaphase in Fig. 9 we observe the two X chromosomes peculiar to female sex. It is generally accepted that Y chromosomes are not involved in sex determination (1); the main sex-determining factor is the ratio: number of autosomes / number of X chromosomes, known also as sex index. When this ratio is lower than or equal to 1:1, the plants are female and when the ratio is bigger than or equal to 2:1, male, respectively. The individuals that have this ratio between 1:1 and 2:1, will be intersexuate. Many experiments on polyploid plant (16) reveal that apart from the plant whose sex corresponds to the sex index, there are some deviant individuals. This fact suggests the existence of maleness and /or femaleness genes on autosomes and that some peculiar combinations of these genes could induce abnormal sexuality. Generally, it is admitted that the maleness genes could be found on autosomes, while femaleness genes – only on X chromosome.

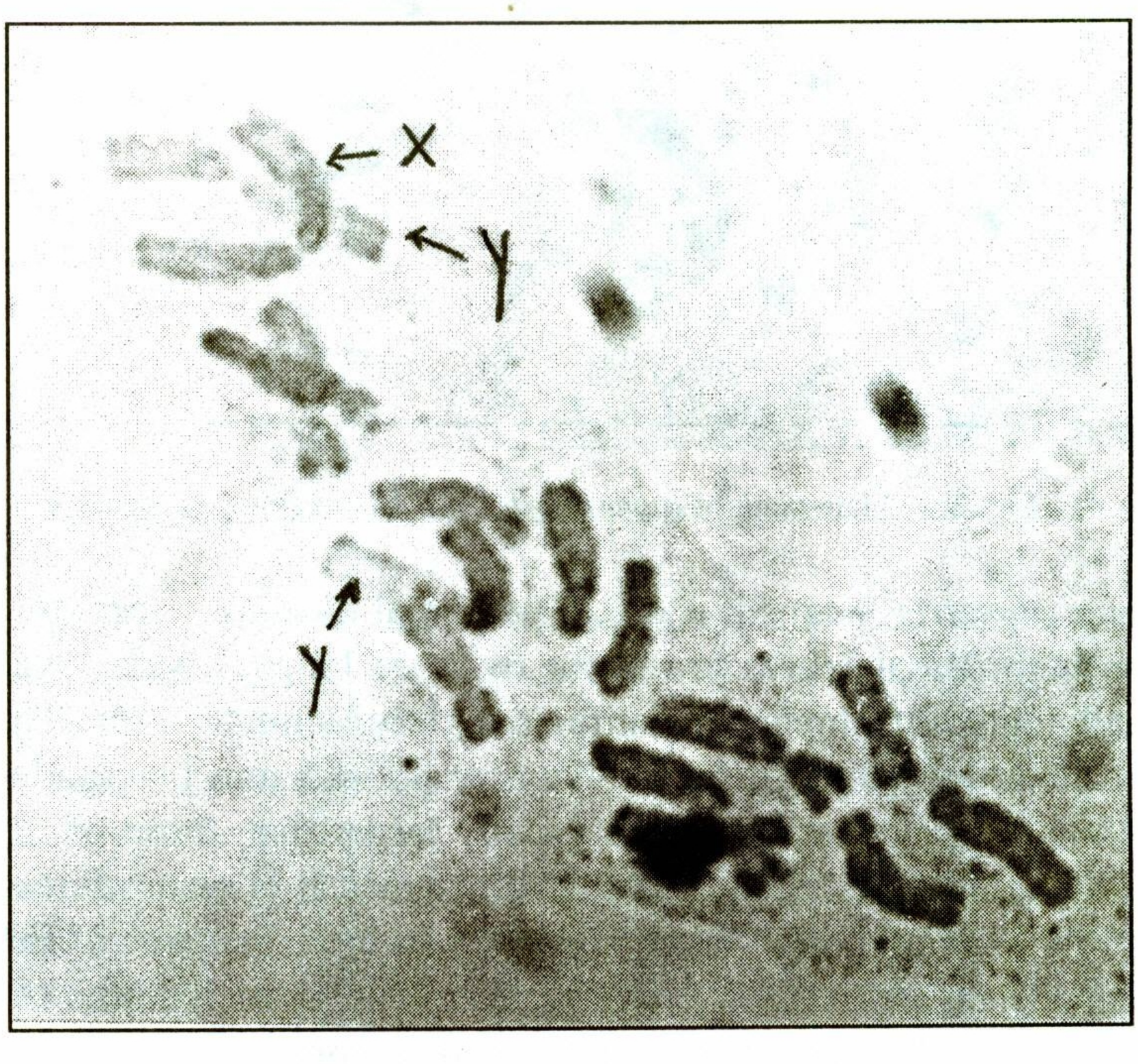


Fig. 3a – Male plant of *Rumex acetosa* L. ssp. *acetosa* – mitotic metaphase.

C banding on female root tips reveals an autosome pattern approximately similar to *R. acetosa* ssp. *acetosa* and X chromosomes with a particular heterochromatic centromere block (Fig. 10).

Fig. 3b – Male plant of *Rumex acetosa* L. ssp. *acetosa* – karyotype.

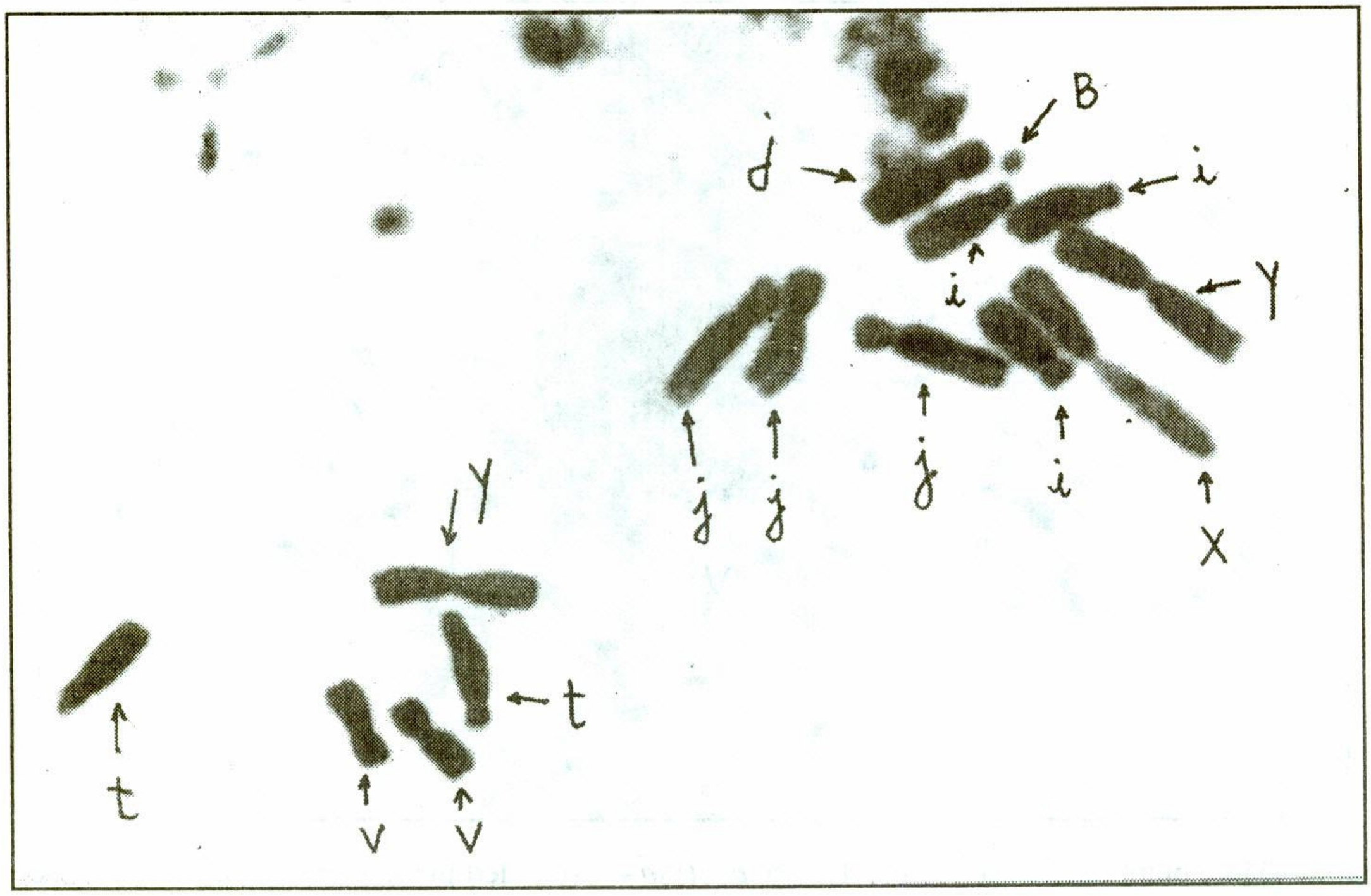


Fig. 4 – Male plant of *Rumex acetosa* L. ssp. *acetosa* – incomplete mitotic metaphasic plate.

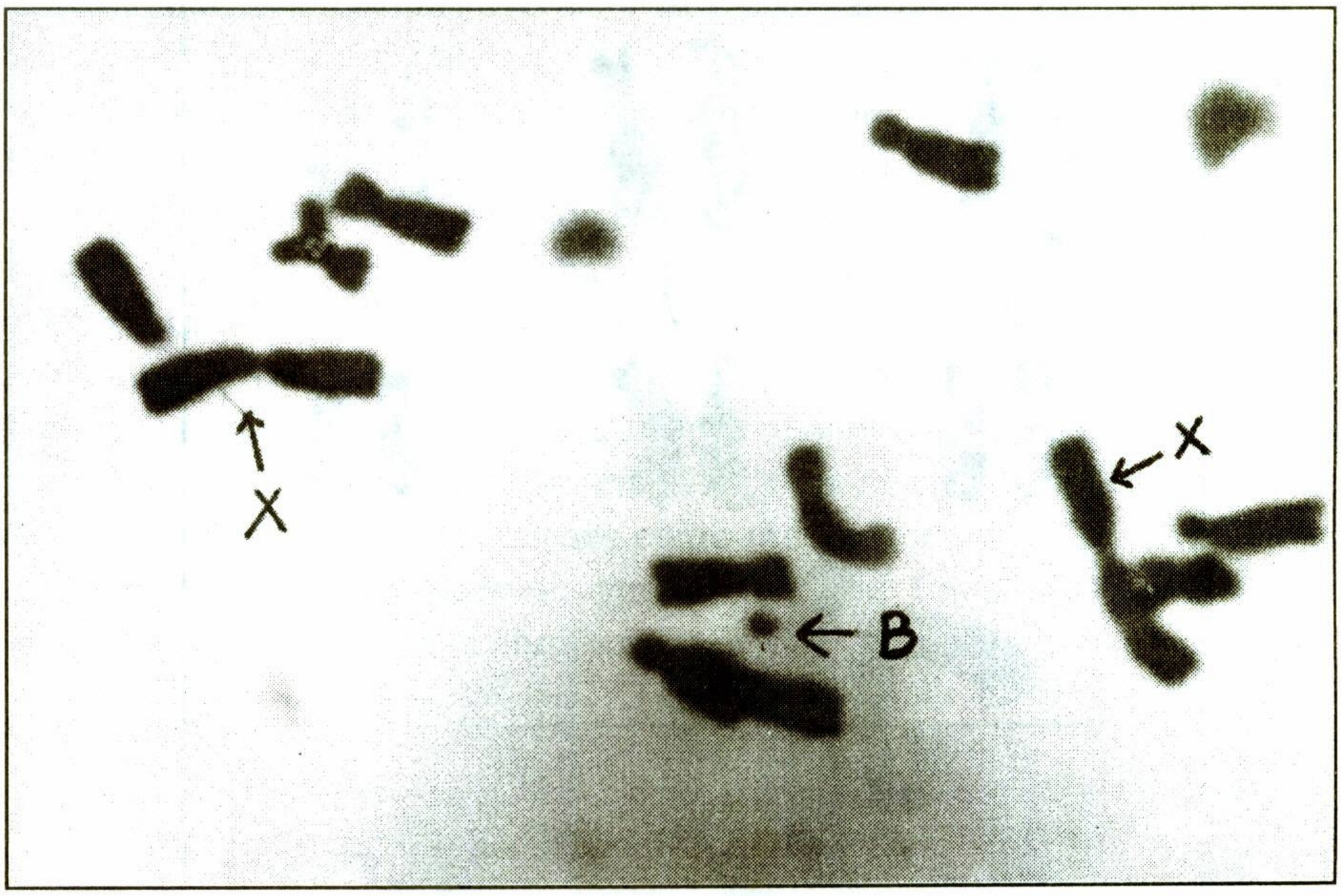


Fig. 5 – Female plant of *Rumex acetosa* L. ssp. *acetosa* – incomplete mitotic metaphasic plate.

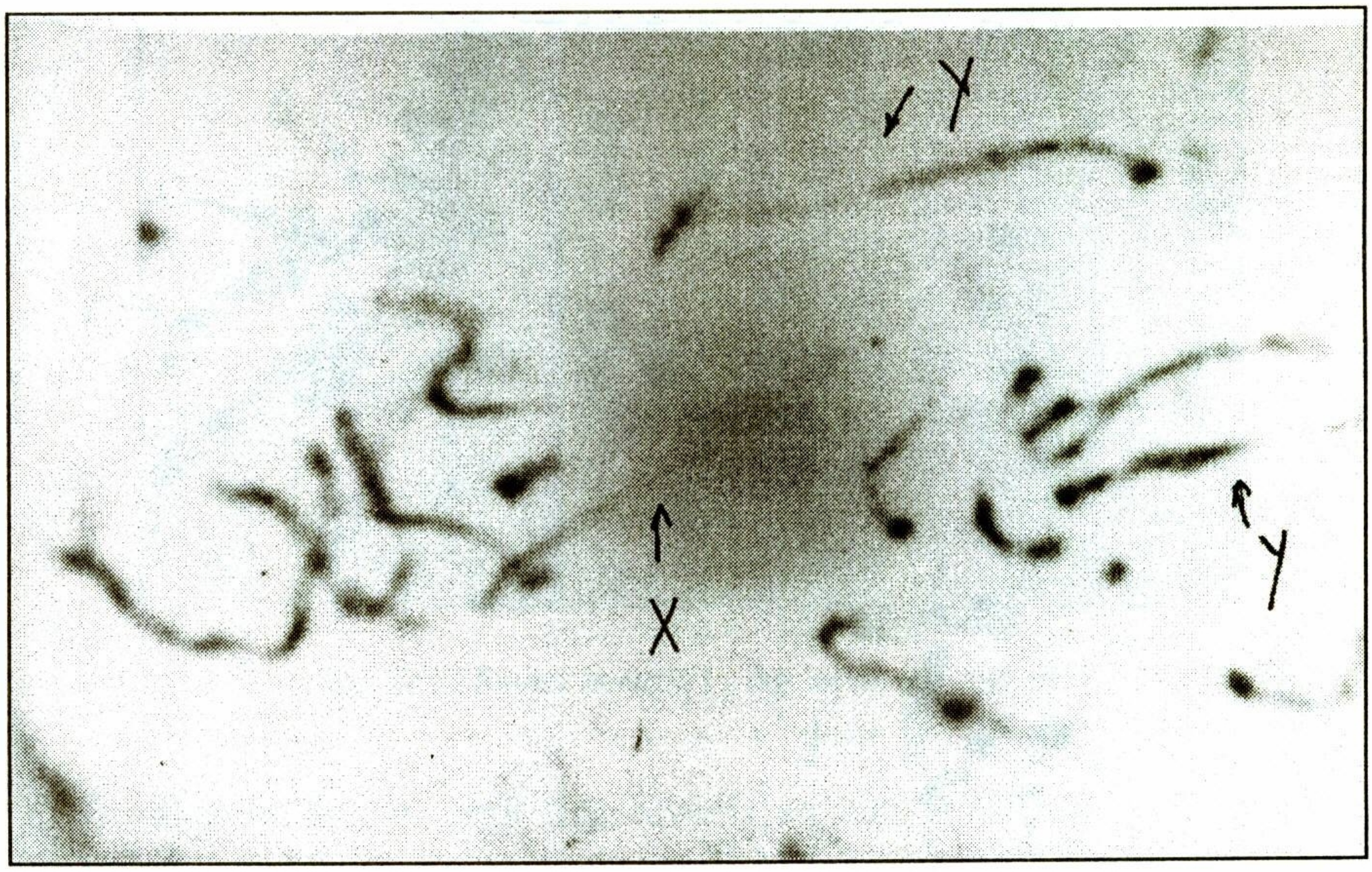


Fig. 6 – Male plant of *Rumex acetosa* L. ssp. *acetosa* – C-banded incomplete mitotic metaphase.

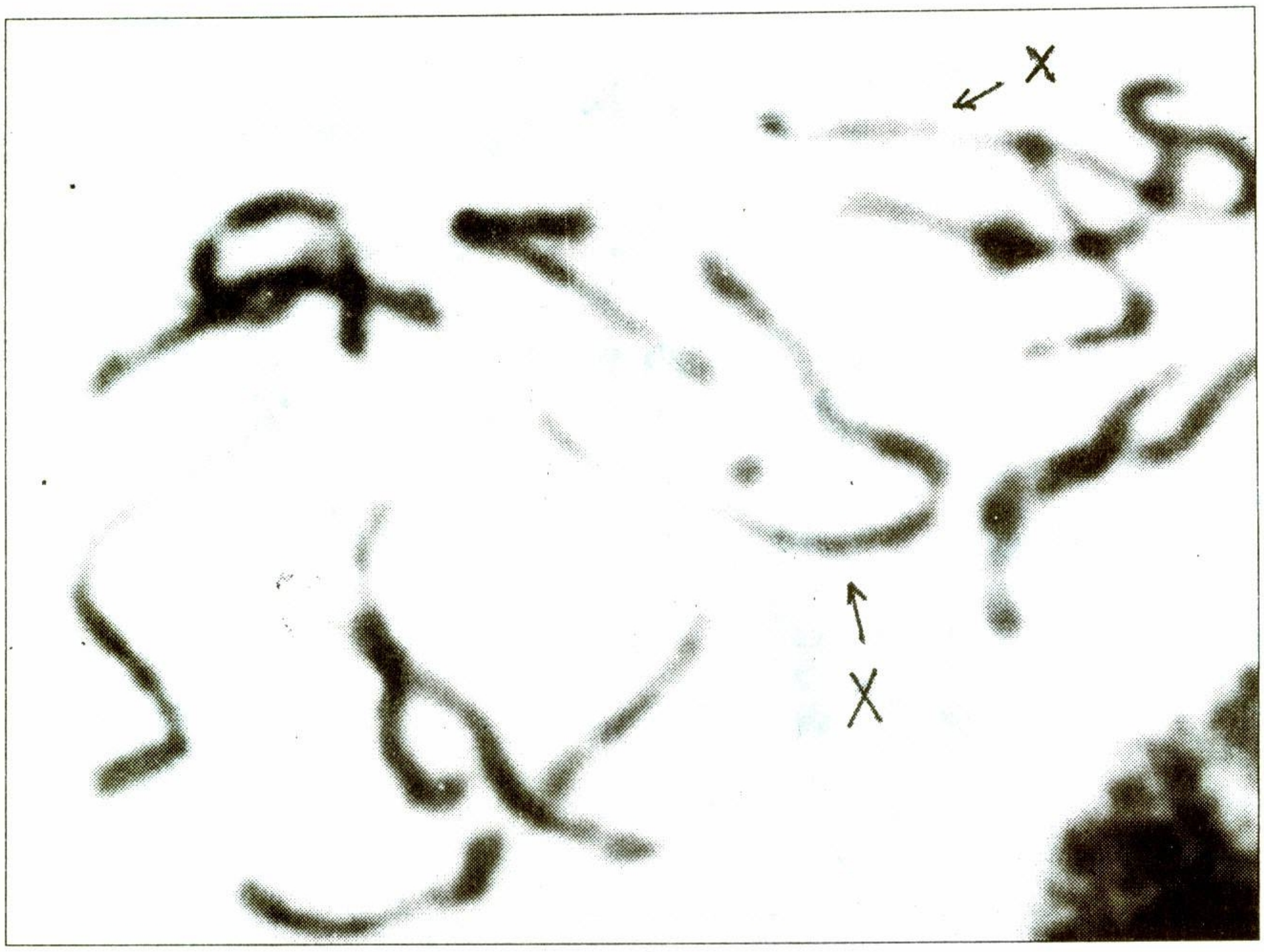


Fig. 7 – Female plant of *Rumex acetosa* L. ssp. *acetosa* – C-banded incomplete mitotic metaphase.

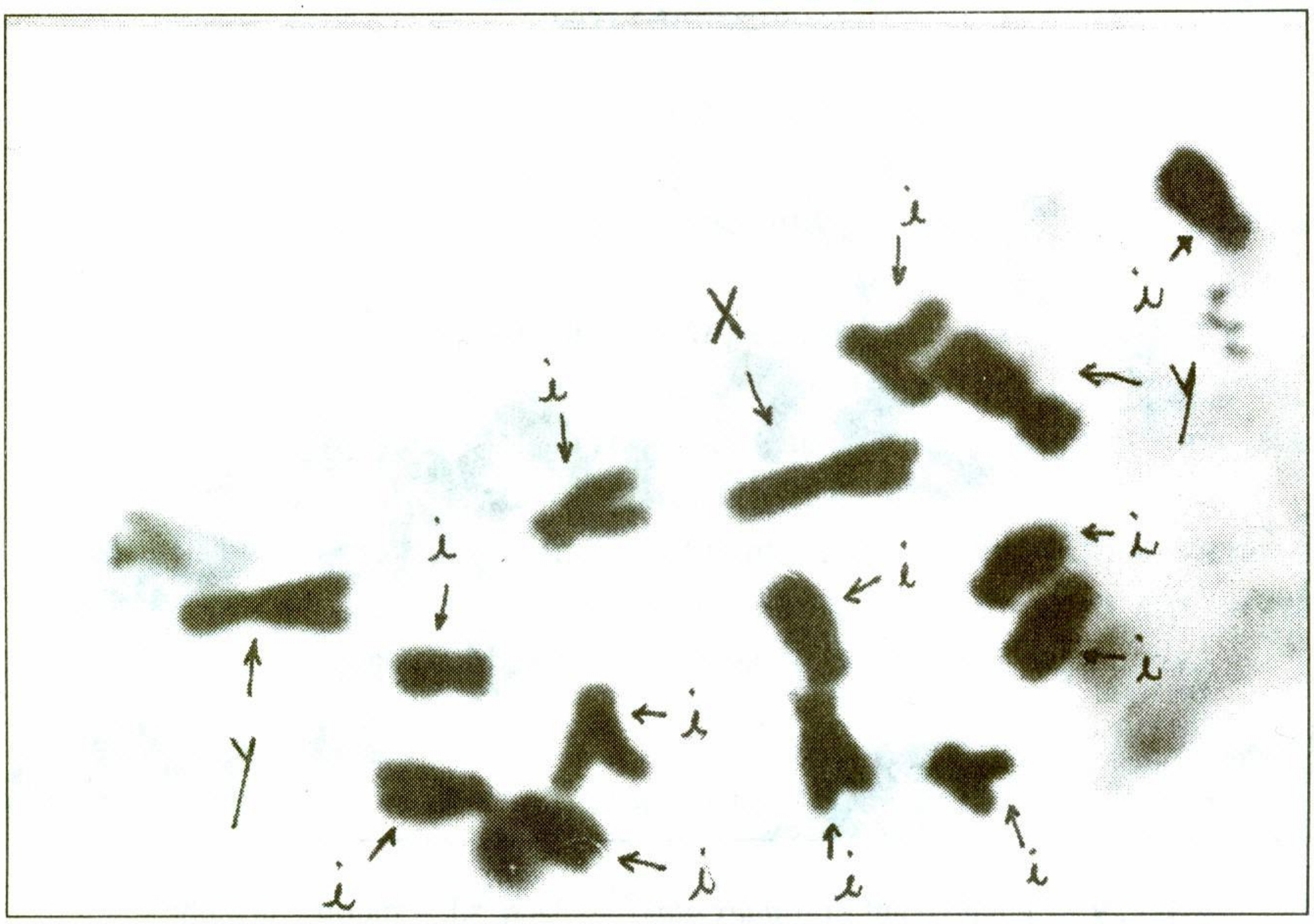


Fig. 8a – Male plant of *Rumex alpestris* Jacq. s.l. – mitotic metaphase;

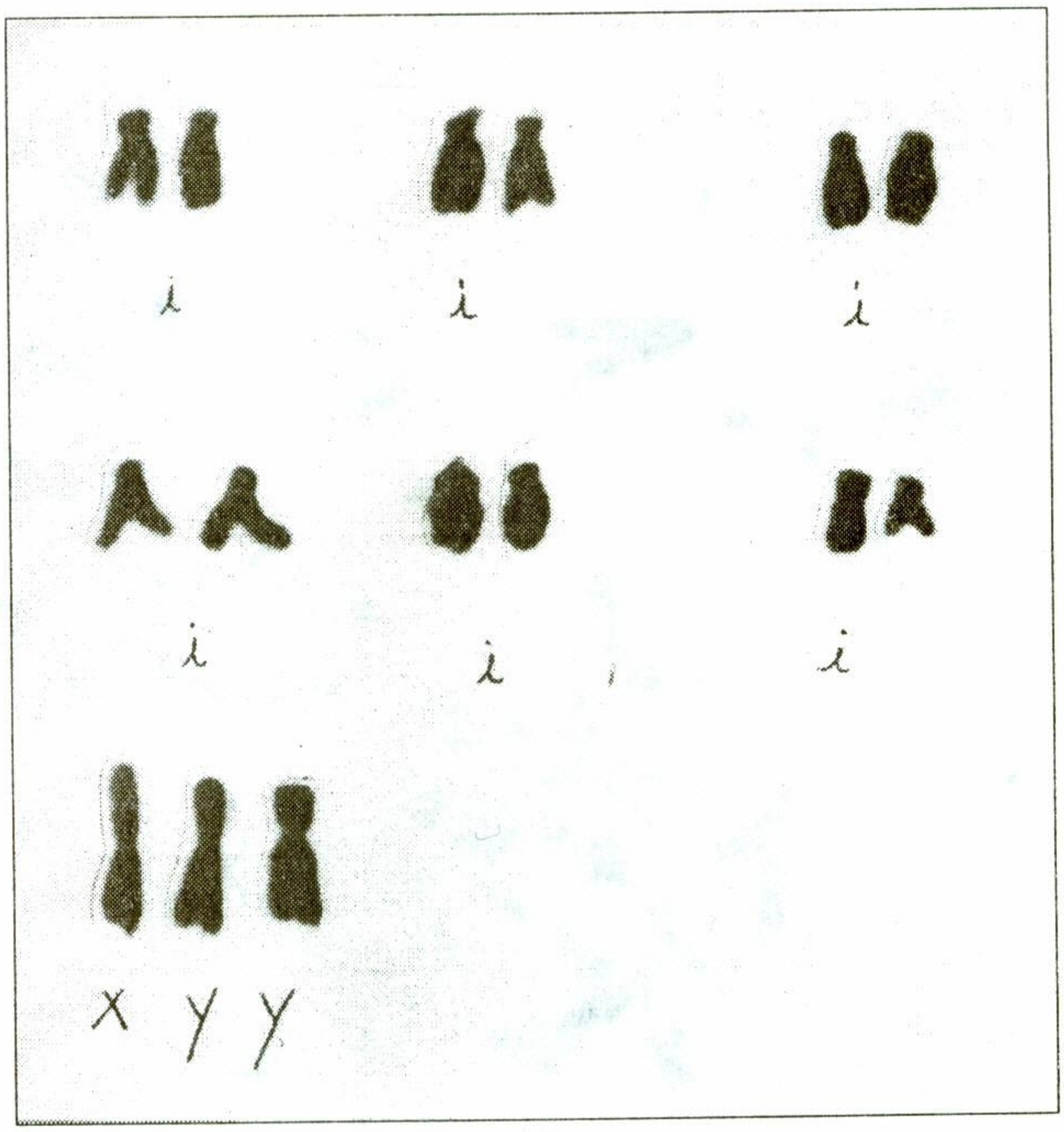


Fig. 8b – Male plant of *Rumex acetosa* L. ssp. *acetosa* – karyotype.

Fig. 9 – Female plant of *Rumex alpestris* Jacq. s.l. – mitotic metaphase.

Fig. 10 – Female plant of *Rumex alpestris Jacq.* s.l. – incomplete mitotic metaphase C-banded.

## CONCLUSIONS

1. We did not observe variations in chromosome number in individuals of dioecious species *R. acetosa* ssp. *acetosa* and *R. alpestris* from different populations. We found the same chromosome number and sex chromosomes as in the literature.

2. *Rumex acetosa* ssp. *acetosa* and *R. alpestris* have similar sex chromosomes properties, the same as cited in the literature for the entire *acetosa* group. The frequency of the particular karyotypes in the various populations was not established since the available data were insufficient on this purpose.

3. C-banding shows both of the Y chromosomes clearly differentiated from autosomes and reveals their heterochromatic nature in *R. acetosa* ssp. *acetosa*. X chromosomes have a heterochromatic centromeric block in *R. alpestris*.

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# HISTOLOGICAL STUDY OF NON-ZYGOTIC EMBRYOS GROWTH *IN OVULO* AND IN THE GYNOGENETICAL CALLUS IN SUGARBEET (*BETA VULGARIS* L.)

MARIA PRISECARU*, PETRE RAICU**, GOGU I. GHIORGHIȚĂ*, ADRIANA FÎNARU*

The cytohistologic studies made on ovaries and ovules after 14 days of cultivation *in vitro* showed the formation of some proembryonic structures with their origin in the egg-apparatus (and/or synergids). Unlike the zygotic embryos, the embryonic structures generated in the absence of fecundation, under our experimental conditions were deprived of suspensor.

The formation of an endosperm-type tissue was not observed in any embryonic shock. The proembryonic structures may generate embryos with a normal evolution in plants or callus in which meristematic regions, embryos and plants are formed secondarily. In spite of the good frequency of induction of the embryos, a large number of that is degenerating in their early stages of development or show teratologic phenomena.

Causes of embryos abortion may be multiple: culture conditions, composition of nourishing media, haploid status on absence of formation/tissue similar to the endosperm, having a trophic and stimulating function in haploid embryos development.

*Key words: Beta vulgaris,* gynogenesis, cytohistological analyses, embryogenic structures, egg apparatus, callus haploid.

## INTRODUCTION

For a long time it has been believed that only the zygot diploid cell, born from two haploid gamets, may give birth to a new individual, by undergoing a succession of embryonic phases. By *in vitro* cultivation a similar evolution to the egg-cells was induced, both of the diploid somatic cells, different from the zygot and the haploid cells from the embryonic sacs or pollen grains (1, 2, 3, 5, 8).

Viable embryos that grow in normal plants are generated during this evolution. The embryogenesis is an essential problem of biology and the aptitude to form complex structures of the embryo type, in the absence of a sexed process is, without any doubt, a remarkable property of the living systems that emphasizes without ambiguity the omnipotence of the vegetal cell (4, 6, 7). The induction of a haploid plant into the culture of ovaries and non-fecundated ovules implies the sporophytic growth of the megaspores megagametophytes in the absence of amphimixis (9,10).

The cytogenetical studies shown in this work and performed in sections on ovaries and ovules of sugarbeet (*Beta vulgaris* L.), cultivated *in vitro*, have confirmed the presence of a cellular activity in the embryonic sacs, in which one or several cells might be involved and they have an apomictic evolution.

## MATERIAL AND RESEARCH METHODS

The gynogenetic development has been observed either directly, in the callus, or by the dissection of ovaries and ovules, under the binocular microscope, either in ovaries and ovules fixed in acetic alcohol (3:1), for 24 hours, sectioned under the binocular lens and coloured with carmine-acetic solution (1%) or in series of sections on fixed material (Navashin-Brunn fixing agent) included in paraffin and cut at 7 thickness with paraffin removal, hydrated and coloured with Erlich hematoxylene.

## RESULTS AND DISCUSSIONS

The cytohistological analyses made on ovaries and ovules fixed after 14 days of culture *in vitro* show the fact that the majority of proembryos structures come from the divisions of the egg apparatus and synergids (Fig. 1, Table 1).

*Table 1*

Distribution of cellular activity within the embryonic sacs of *Beta vulgaris* L. after 14 days of cultivation

| Genotype | No. of sectioned ovaries | No. of embryonic sacs with cellular activity | Egg apparatus | Egg apparatus and synergids | Antipodal cells | Polar nuclei |
|---|---|---|---|---|---|---|
| Bârsa | 20 | 15 | 10 | 5 | – | – |
| Decor | 10 | 3 | 3 | – | – | – |
| $CV_{14}$ | 10 | 5 | 4 | 1 | – | – |
| $SG_{29}$ | 10 | 5 | 2 | 3 | – | – |

Subsequently, these structures may develop into embryos (Fig. 2) or callus (Figs. 3, 4). Thus, within 30 studied ovaries belonging to Bârsa, Decor varieties and the $CV_{14}$ diploid line and 20 ovules of Bârsa variety and the $SG_{29}$ tetraploid line proembryos formations have been identified in different stages of evolution, in the area of the egg apparatus.

In some sections the synergids are visible, confirming thus the origin of proembryos in the egg apparatus (Fig. 1).

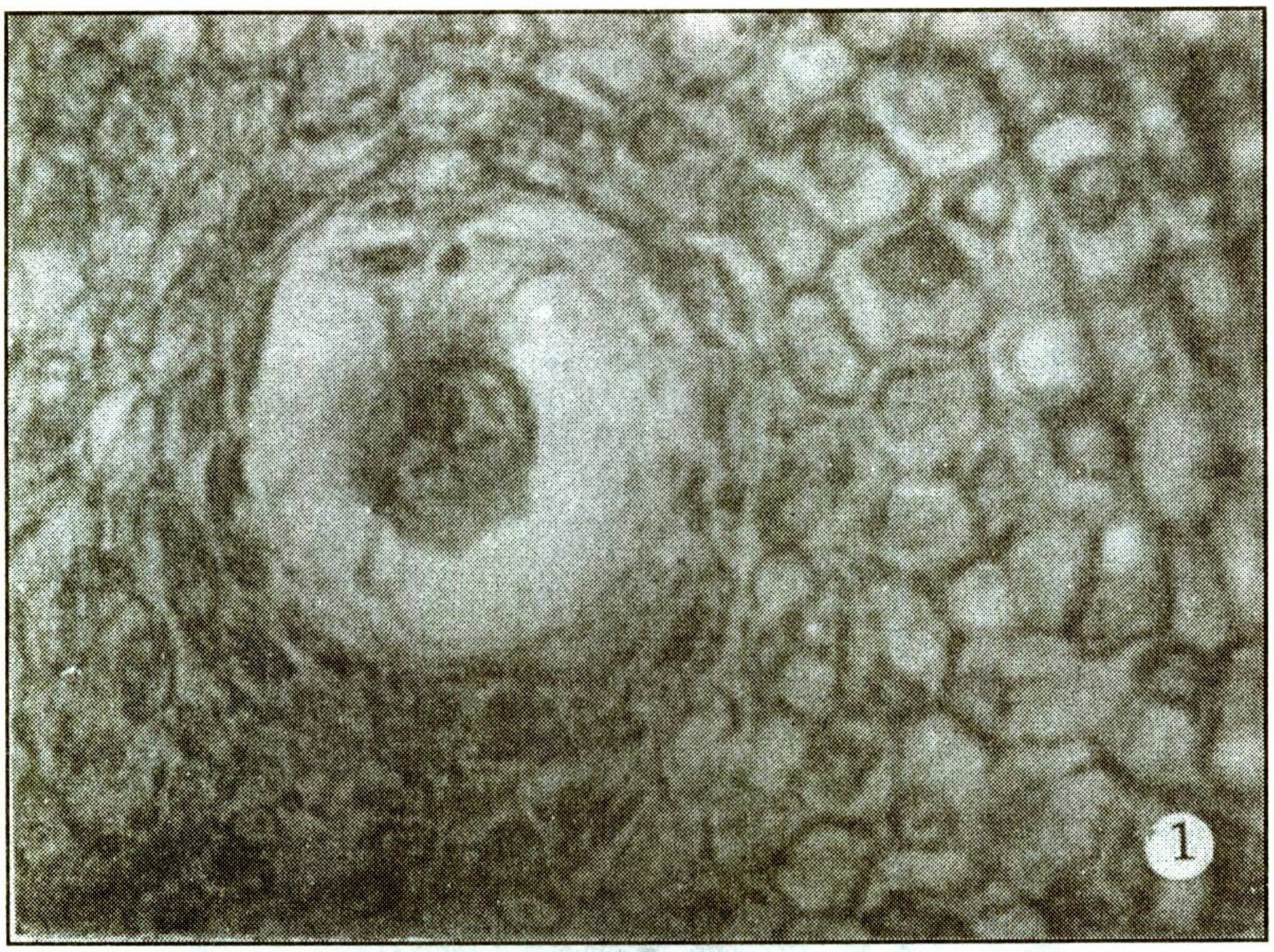


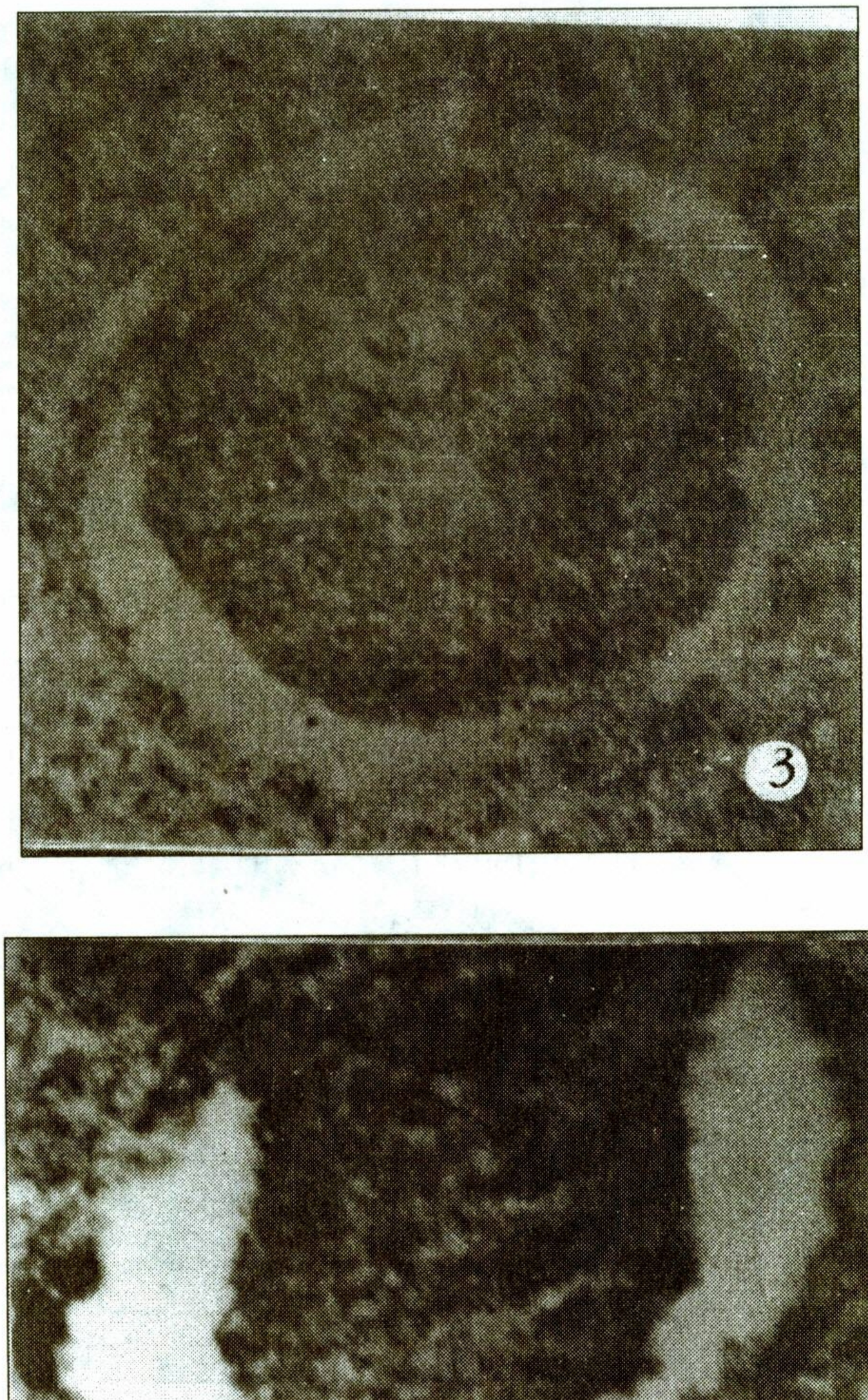

Fig. 1 – Egg apparatus largely magnified and under division process.

Fig. 2 – Normally grown embryo, present in the embryo sac.

Part of the proembryos of globular or sharpened forms have developed into the callus or embryos.

The concomitant presence of several embryonic structures, grown together (2–3) in the same embryonic sac, has been found in some of the sections, the so-called polyembryos in the micropylar end by the potential participation of the whole egg apparatus.

No proembryos formation was found in the antipodal apparatus region or polar nuclei. No cellular structures similar to the endosperm have been found near the proembryos. The newly formed proembryos unlike the zygotic ones did not show any suspensor (Fig. 3).



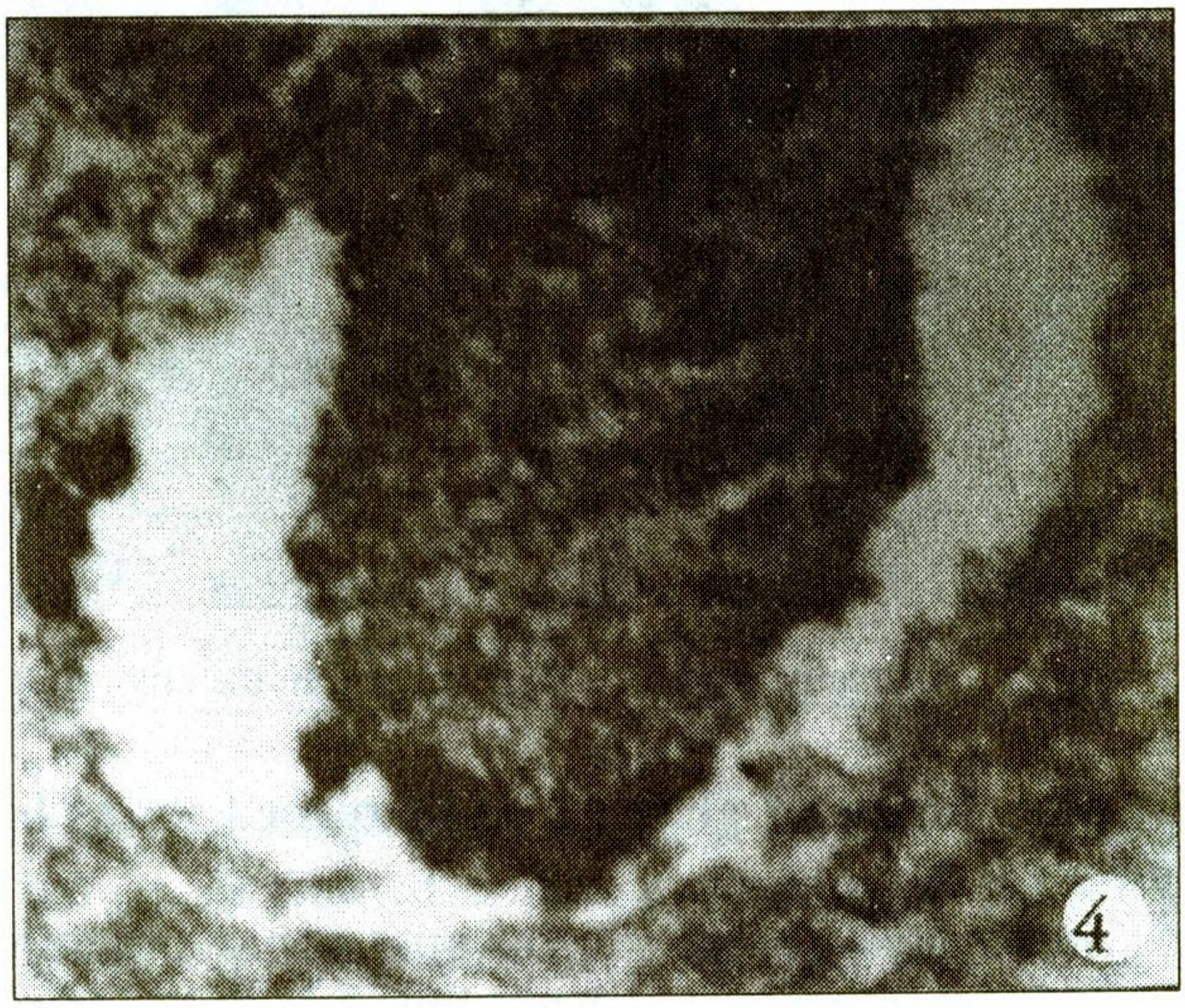




Fig. 3, 4 – Proembryo with evolution into callus.

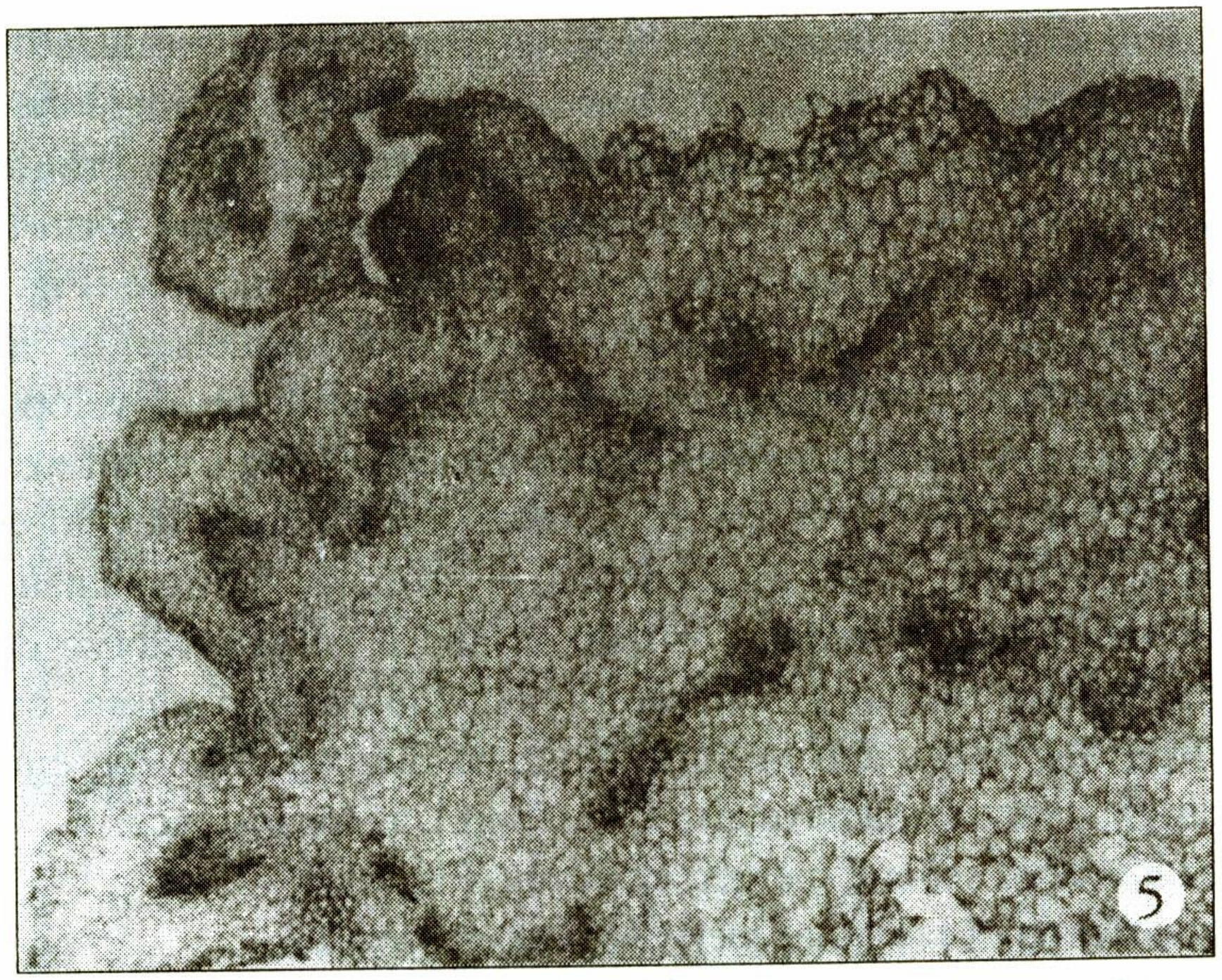



Fig. 5 – Gynogenetic callus in which embryogenic structures have developed.

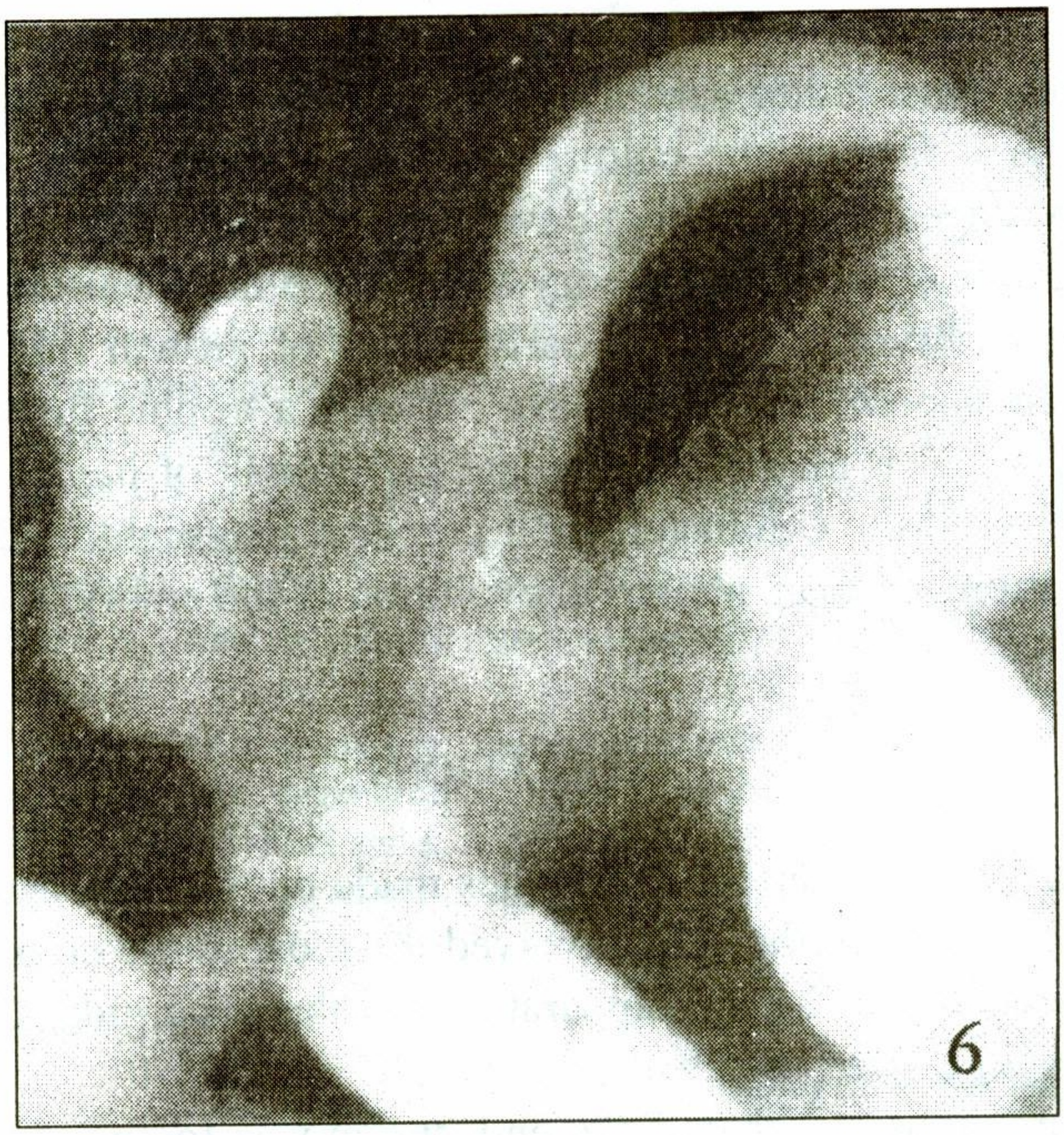



Fig. 6 – Simultaneously grown embryos and developed within the same embryo sac.

Microscopic analyses made on the gynogenetic callus produced by a part of the ovaries and ovules after 4 weeks of cultivation rendered evident the presence of an intensive process of meristematic center formation. By proliferation, the meristemoids give to the callus a microglomerular aspect and form the globular embryos or polarized structures visible from the callus surface too (Fig. 5).

In our experimental conditions, even if the frequency of induction of gynogenetic growth to some genotypes was good, still the development of the embryonic conversion into plants was accomplished only to a small extent. A large number of the formed embryos were aborted during their development in the differentiating media. A lot of proembryos in sections showed changes of their normal structure, differentiation in suspensor and a proper embryo did not form degenerating without reaching full maturity. In the embryo phase, both in those developed from the callus and in those emerged directly from the embryonic sac cells, teratologic phenomena have also been found, as for instance:

- appearance of several merged embryos (Fig. 6)
- degeneration of radicular meristems or cotyledons.

Such teratologic forms are considered to appear due to some deficiencies in the endogenous synthesis of the cytokinins or to some mineral or hormonal disbalance of the culture media.

We have to consider that the genetic factors are categorically involved, since the chromosomic constitution does not always allow a normal cytodifferentiation. Besides the genetic factors, the culture media conditions exert a great influence both in the early stages of the embryo development and during its maturation.

Following our observations we try to advance the idea that the high rate of mortality of the embryos since their early stages of growth might be due to the absence of the endosperm that could have not only a trophic function provided by the culture media, but it may also stimulate the growth of haploid embryos. Such absence noticed in all the cytohistological preparations might lead to the lack of a suspensor formation and to an abnormal evolution of the embryonic growth.

## CONCLUSIONS

According to the cytohistologic studies made on the sugarbeet embryonic sac it has been established that the cells involved the most in the embryonic apomictic development *in vitro* are the egg apparatus and the synergids, the former being involved to a higher degree (64.2%).

No structure similar to that of the endosperm was found and the proembryos did not have a suspensor. Depending on the culture media conditions and the chemical composition of the nourishing media the proembryo structures developed

either into normal embryos that produced plants, or into embryogenic callus that generated secondary meristematic centres, embryonic structures and plants.

In spite of the efficient formation of the gynogenetic embryologic structures (Table 1), since their subsequent evolution was not quite favourable, because they degenerated their early stages. Many embryos presented teratologic phenomena, generating abnormal plants. The causes of the abortion of gynogenetic proembryos may be multiple: the culture conditions, the composition of nourishing media, haploid status or the occurrence of chromosomic perturbations. A major cause could be the absence of an endosperm formation that might perform not only a trophic function but also a stimulating one in sustaining the normal development of the haploid embryos. It is possible that the problem of haploid gynogenetic embryos survival in angiosperms be solved partially by finding some ways to stimulate the polar nuclei divisions at the same time as the formation of an endosperm-type tissue.

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# THE MORPHOGENETIC REACTION OF SOME *STACHYS SIEBOLDII* MIQ. EXPLANTS IN *IN VITRO* CULTURES

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IRIS STĂNESCU**

The shoot tips, nodes, stem fragments, roots and leaves of *Stachys sieboldii* Miq. cultivated on MS (1962) medium supplemented with 2.4-D, BAP and 2.4-D, or BAP, NAA and 2.4-D, generated a compact callus, of a grey-green colour, with low proliferation capacity. The nodes and shoot tips, inoculated on media supplemented with BAP, kinetine, BAP and NAA, favoured callogenesis with frequent multiple shooting. On MS medium lacking hormones, or with reduced quantities of NAA, the shoots generated roots. The neoplantlets accommodation on septic conditions was an easy and efficient process and the field transfer of the regenerants was performed without significant losses.

## INTRODUCTION

Modern therapeutics of the last quarter of this century proved the evidence and value of some substances with iridoid structure. The substances are extracted from the tuberized secondary roots of *Harpagophytum procumbens*, species originating from the south of Africa (2, 4). The iridoids of this plant (of harpagid type) have a marked antirheumatic, anti-inflammatory and analgesic action. The outstanding therapeutical value of the mentioned products led to an increase of the needs of the pharmaceutical industry regarding the raw material offered by this species. As in field culture this species has not been very productive up to now (not even in its original area), specialists tried to identify other vegetal sources containing similar substances.

It has been noticed that iridoids of the harpagid type are contained by some species of the *Stachys* (1, 5). Our attention focused on *Stachys sieboldii* Miq., *Lamiaceae* family. Also known as *S. tuberifera* Naud., original from North China, used as a nourishing product (its tubers contain 14% stachyose in fresh matter) and in traditional medicine. Our investigations aimed at knowing the behavior of this species and its biosynthetical potential in conventional and *in vitro* cultures. Some preliminary results were published in previous works (3, 6, and 7).

## MATERIAL AND METHODS

The initiation of *Stachys sieboldii in vitro* cultures was performed in 1991 in the Botanical Gardens from Chişinău and the "Stejarul" Research Centre, Piatra

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Neamț. We used as explants shoot tips of glass house cultivated plants and field cultivated plants, respectively. The explants were sterilised in $HgCl_2$ solution (0.01% 15 and 12 minutes respectively) followed by repeated washings in sterile distilled water. The sterile explants were inoculated on MS medium (1962) in 100 ml Erlenmayer flasks.

In the Chişinău experiments MS was supplemented with gibberelic acid, kinetine, saccharose (60 g/l) and agar (6.0 g/l); in the Piatra Neamț experiments, MS medium was supplemented with BAP, BAP and NAA, kinetine and NAA or 2.4-D, saccharose (25 g/l) and agar (8.0 g/l). The cultivated flasks were incubated in a climatized room (23–25°C, 2500 lx luminosity, continuous illumination).

The experiments continued in the following years at the "Stejarul" Research Centre-Piatra Neamț, where we used sterile explants from *in vitro* regenerated neo-plantlets. We tested different types of explants: shoot tips, nodes with leaves, internode fragments, leaves, roots and callus. The explants were inoculated on diverse hormonal variants of the MS medium (Table 1) in the 100 ml Erlenmayer flasks. We added saccharose (25 g/l) and agar (7–8.5 g/l) to the nutritious medium. The cultivated flasks were placed in the same conditions. The transfers were performed at intervals of 5–8 weeks.

*Table 1*

Hormonal variants of MS medium used for the *in vitro* cultivation of *Stachys sieboldii* species

| No. | The variant | The growth regulators (mg/l) | | | | | |
|---|---|---|---|---|---|---|---|
| | | BAP | Kin | IAA | IBA | NAA | 2.4–D |
| 1. | B | 2.0 | – | – | – | – | – |
| 2. | BA | 2.0 | – | 1.0 | 0.5 | – | – |
| 3. | BB | 1.0 | – | – | – | – | – |
| 4. | BD | 1.0 | – | – | – | – | 1.0 |
| 5. | BD-2 | 0.5 | – | – | – | – | 2.0 |
| 6. | BDN | 0.5 | – | – | – | 0.5 | 0.5 |
| 7. | BK-1 | 2.0 | 1.0 | – | – | – | – |
| 8. | BK-2 | 1.0 | 0.5 | – | – | – | – |
| 9. | BN | 1.0 | – | – | – | 0.5 | – |
| 10. | D-2 | – | – | – | – | – | 2.0 |
| 11. | KD | – | 1.0 | – | – | – | 0.5 |
| 12. | KN | – | 1.0 | – | – | 1.0 | – |
| 13. | N | – | – | – | – | 0.5 | – |

Along the investigations we made evaluations regarding the influence of the hormonal balance on the number of shoots generated per explant, on the fresh and dry biomass accumulated per flask in a certain period of time, etc.

The *in vitro* regenerated plants were accommodated to the septical conditions either in the hydroponics systems or in the flowerpots with sterile soil and were then transferred to the experimental field of the Piatra Neamț "Stejarul" Research

Centre. The observations regarding the morphogenetical reaction of the explants and the biomass accumulation are in Tables 2 and 3.

## RESULTS AND DISCUSSIONS

The basic reaction of the nodes with the afferent leaves, or of shoot tips inoculated on B, BA, BB, BK and BN formulae was caulogenesis. A green-gray, compact, small callus formed at the contact with the culture medium. On medium B, multiple shooting was well represented. The presence of NAA somehow intensified callus generation although the basic process remained caulogenesis. The callus formed on BN around the node or shoot tip (compact, dark-green) presented organogenetic zones which developed either buds and shoots (more frequently) or roots. At the same time, we noticed that along with ageing (after 3–4 years of continuous *in vitro* cultivation) the caulogenetic capacity of nodes and shoot tips on BN was significantly reduced, yet shooting from axilary buds is well represented. On BA, BB and BK the reaction was similar but much more reduced in intensity.

On KN, the nodes and shoot tips generated either neo-shoots from axilary buds or callus. The shoots proved a better development on B, BA and BK, but without uniformity, presenting different sizes. The callus generated on this medium (compact, green-gray, reduced proliferation) frequently formed – on its surface – buds and shoots.

The auxine added to the nutrition medium seems to be decisive for the morphogenetic reaction of the tested explants. When we replaced NAA with 2.4-D in the medium, irrespective of the cytokinine used (BAP or Kin) inhibited caulogenesis. The nodes and shoot tips placed on the media with 2.4-D generated a compact, grey-green callus, with a rugged aspect and a low proliferation capacity. If to the mentioned medium we had added NAA (the BDN variant), the basic reaction would have remained the same but the callus cells would have registered a more intense proliferation.

On the media with cytokinines, the internodes had no reaction. In the presence of auxines or a combination of cytokinines + auxines, at the surface of the internodes fragments, a thin stratum of grey-green callus was formed, the multiplication speed being reduced. On KN, along with the callus, especially generated at the ends of the fragments, a fine network of roots formed at the non-callused contacting surfaces of the explants with culture medium.

The root fragments inoculated to media with 2.4-D or BAP and 2.4-D had a reaction similar to that of the stem fragments (a green-gray, compact callus, with low proliferation) (Table 2).

Cultivated on media supplemented only with cytokinine, the young leaves or leaf-fragments had no other reaction than an increase of their dimensions. A similar

behaviour was registered by these explants on a medium supplemented with IBA. On the D-2, BD, BDN and KD, the leaves grew and generated on the whole surface, or only on certain areas, a compact, green-gray callus with low proliferation – but superior to that originated from the stem and root fragments (especially on BDN). On BN, and in a smaller ratio on KN, the leaf fragments (especially in the petiole area) formed a green, compact callus, with an average proliferation capacity, which later produced either shoots or roots; on KN rhizogenesis had a superior intensity (Table 2).

*Table 2*

The morphogenetic reaction of *Stachys sieboldii* explants on different culture media

| **The explant** | **Hormonal formulae** | **Basic reaction of explants** | **Intensity of reaction** | **Observations** |
|---|---|---|---|---|
| **1** | **2** | **3** | **4** | **5** |
| Shoot tips nodes with afferent leaves | B | multiple shooting | ++++ | high biomass quantity |
| | BA | multiple shooting | +++ | rhizogenesis |
| | BB | multiple shooting | +++ | rhizogenesis |
| | BD | callusogenesis | + | green-gray compact callus |
| | BD-2 | callusogenesis | ++ | green-gray compact callus |
| | BDN | callusogenesis | ++ | callus formed at the contact of the explant with culture medium |
| | BK-1 | multiple shooting | +++ | rhizogenesis |
| | BK-2 | caulogenesis | + | feeble shoots, sporadic rhizogenesis |
| | BN | multiple shooting rhizogenesis | +++ | organogenetic callus at the contact of the explant with culture medium |
| | KN | caulogenesis | ++ | sporadic callusogenesis |
| Shoot tips nodes with afferent leaves | KD | caulogenesis | + | compact, green-brown callus |
| | N | caulogenesis | ++++ | vigorous shoots with roots (inclusive aerial roots) |
| Internode fragments | B | no reaction | | |
| | BD | callusogenesis | ++ | green-grey, rugged. compact callus |
| | KD | callusogenesis | + | campact callus, cream-green coloured |
| | KN | callusogenesis | + | rhizogenesis |

*Table 2 (continued)*

| 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|
| Leaf fragments | B | growth of dimension | ++ | |
| | BD | callusogenesis | ++ | compact, green callus |
| | BDN | callusogenesis | +++ | compact, green-grey callus |
| | BN | callusogenesis | +++ | compact callus at the level of petiole; the callus generated buds, shoots and roots |
| | D-2 | callusogenesis | ++ | compact, green-grey callus |
| | KD | callusogenesis | ++ | compact, green-grey callus |
| | KN | callusogenesis | ++ | compact callus, developed at the petiole level organo-genesis; intense rhizogenesis |
| Root fragments | BD | callusogenesis | ++ | compact, rugged, green-coloured callus |
| | BD-2 | callusogenesis | + | compact, green-grey-coloured callus |
| | D-2 | callusogenesis | + | compact, green-grey-coloured callus |

Legend: + reduced reaction; ++ moderated reaction; +++ good reaction; ++++ very good reaction.

*Table 3*

The variation of some quantitative indices of *in vitro* cultures of *Stachys sieboldii* depending on the hormonal balance used in the nutrition medium (inoculation 30.03.1995 – harvesting 12.05.1995)

| Sample No. | Hormonal formulae | No. shoots/ explant | Biomass (g/flask) fresh b. | dry b. | Dry matter content (% fresh matter) |
|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 |
| 1. | BN | 7.2 | 6.5069 | 0.3945 | 6.063 |
| 2. | BN | 8.6 | 5.6211 | 0.4751 | 8.452 |
| 3. | BN | 12.0 | 7.7990 | 0.4280 | 5.428 |
| 4. | BN | 8.6 | 7.6005 | 0.4126 | 5.428 |
| 5. | BN | 9.4 | 5.3736 | 0.3917 | 7.289 |
| 6. | BN | 8.6 | 6.0520 | 0.4201 | 6.941 |
| 7. | BN | 9.0 | 6.5470 | 0.3079 | 4.702 |
| Average | BN | 9.6 | 6.5000 | 0.4043 | 6.344 |
| 1. | B | 11.4 | 4.0725 | 0.2424 | 5.952 |
| 2. | B | 8.1 | 6.3730 | 0.3030 | 4.754 |
| 3. | B | 9.9 | 8.7003 | 0.3633 | 4.175 |
| 4. | B | 6.7 | 5.6800 | 0.2582 | 4.546 |
| Average | B | 9.2 | 6.2064 | 0.2917 | 4.875 |

The results obtained by the *S. sieboldii in vitro* cultures allow us to appreciate that this technique is not advisable to be used for biomass callus formation. For biologic or planting material (on great surface) multiplication and to obtain the seeding material necessary to cultivate the plant on large surfaces one might either use direct organogenesis (from nodes and shoot tips), or indirect organogenesis (via "callus"), preferably using young leaves. The most suited media in this respect are the ones supplemented with BAP or Kinetine, associated with NAA. On these media one may directly obtain neoplantlets. The nodes and shoot tips generate numerous other shoots from axilary buds on medium supplemented with BAP resulting a massive rooting if transferred on MS without phytohormones, or when a reduced quantity of NAA (0.1 mg/l) is added. Some aspects of *Stachys sieboldii in vitro* culture are presented in the photos (Fig. 1).

An interesting peculiarity of this species is the differentiation of overgrown (adventitious) roots from every node (Fig. 1d), a fact that contributes (along with the raised pubescence of stems and leaves) to a rapid and without losses accommodation of the neoplantlets to the septic conditions (if a relatively high air humidity is assured). The neoplantlets accommodation was successful both on a hydroponics system and in pots with sterile soil. The field transfer of the regenerants is without problems and losses. Some neoplantlets formed small tubers already in pots with soil. During the regenerants transfer from pots into field we noticed that some had tubers of forms and colours different from those directly transferred into the field: longer and thinner, white; when the tuber developed in air it got a green colour (similar to potatoes). If the option is for direct organogenesis to obtain seeing material and the initial material is sterile, the whole process – from explant to accommodated neoplantlets – takes 1.5–2 months.

During the investigations, we also made some observations regarding the shoots generated per explant, fresh and dry biomass on phial, depending on the hormonal balance of the medium (Table 3). We noticed that on the medium with BAP (2 mg/l) there appears an average of over 9 shoots/explant, the fresh mass on flask was between 4.07 and 8.70 g, with an average dry mass of 4.85% (of the fresh substance). Similar results were obtained on BN: an average of over 9 shoots per explant, a fresh mass/flask between 5.37 and 7.79 g, with 6.34% dry substance. The shoots obtained *in vitro* presented a pronounced variability of the dimensions, from 1.0 to 7.0 cm during a passage (about 5 weeks).

*In vitro*, *Stachys sieboldii* Miq. showed a frequent appearance of the fasciation phenomenon. In the conditions of a great hormonal supply – longer time – the hyperhydry phenomenon appeared. This was easily corrected by transferring the explants on MS medium without hormones and a more pronounced solidification of the nutrition medium (over 8.0% agar). Unlike other species maintaining the plant *in vitro* for a long time (about 5 years) it did not lead to a significant reduction of the explant morphogenetic reaction.

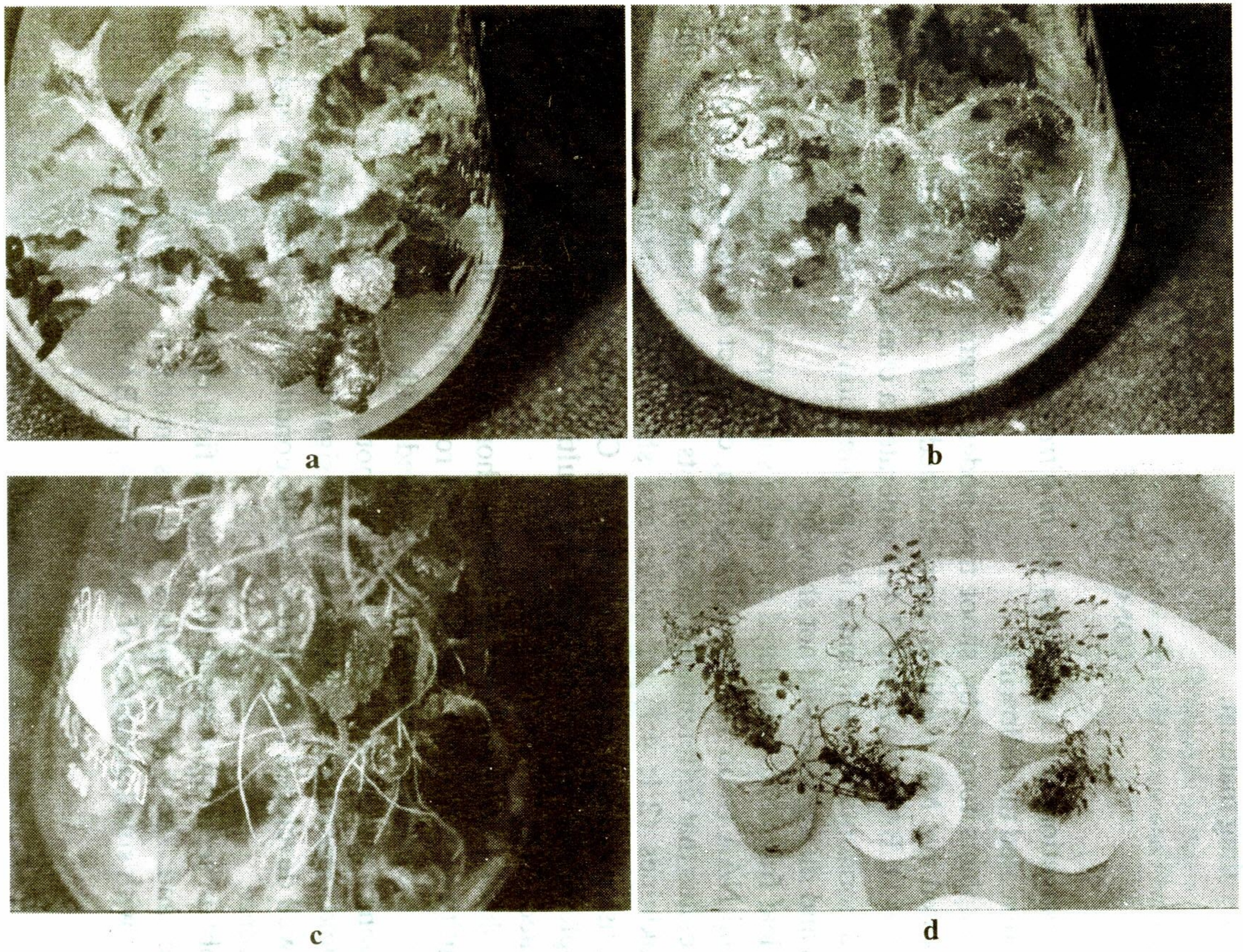

Fig. 1 – Aspects of *Stachys sieboldii* Miq. *in vitro* cultures: a) the aspect of the callus and shoots generated on medium supplemented with BAP and NAA; b) neoplantlets obtained on MS without hormones; c) overground roots regenerated at shoot nodes; d) neoplantlets accommodated in the hydroponics system.

The neoplantlets transferred into the experimental field of the "Stejarul" Research Centre had a good development, generated new plantlets through vegetative multiplication (tubers) and manifested a high resistance to drought and heat ( the species probably being a xerophyte). The species may, without problems, be introduced into culture in our country and the *in vitro* technologies may assure the necessary seedling material.

## CONCLUSIONS

Our investigations for 5 years, on the *in vitro* behaviour of *Stachys sieboldii* Miq. species revealed the following:

1. The explants formed by shoot tips, nodes, fragments of stem, roots and leaves, cultivated on MS medium supplemented with 2.4-D, BAP and 2.4-D, kinetine and 2,4-D, BAP, NAA and 2.4-D generated a characteristic callus, rugged aspect, with a horizontal growth and a low proliferation capacity. On these media caulogenesis and rhizogenesis did not show up.

2. The leaf fragments inoculated on media supplemented with BAP and NAA or kinetine and NAA (ratio 1:1) formed a green, compact callus, especially in the petiole area, callus that differentiated either shoots or roots. In case of a favourable ratio of cytokinines (2:1; 4:1) the nodes and shoot tips generated a callus of reduced dimensions, shoots and frequently roots. On these media, as well as on the medium supplemented only with BAP, the multiple shooting phenomenon was well represented.

3. The shoots inoculated on MS without hormones, or in the presence of a reduced quantity of NAA (0.1 mg/l), generated roots. The accommodation of the neoplantlets to the aseptic conditions was realized, with the same efficiency, both in the hydroponics system and in the sterile soil pots.

4. In field transfer of the regenerants no significant losses were registered. The plant is well developed in the pedo-climatic conditions of our country and may be used in culture.

5. To obtain seedling material necessary in field cultures, we recommend the use of node and shoot tip explants and as nutrition medium MS (1962) supplemented with small quantities of BAP, NAA, or BAP and NAA.

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# HAPLOID PLANTS INDUCTION FROM OVULES OF BEET (*BETA VULGARIS* L.) WITH DIFFERENT DEGREES OF PLOIDY

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The haploid plants of the beet can be successfully obtained from the young ovules on the top of inflorescence. The morphologic aspect and the ovule and ovaries association are very important factors in the setting off the process of the organogenic callous growth. The culture medium used by Van Geyt and Jacobs (1985) was enriched by BAP, IAA and 2,4 – D in different ratios depending on the targeted phenologic moment. The genotype has a decisive effect on the haploids. There are beet genotypes that cannot yield organogenic calloses from ovules.

*Key words: Beta vulgaris,* breeding, development *in vitro,* haploid plants.

## INTRODUCTION

The advantages of the techniques of *in vitro* culture by the induction of gynogenesis, with the help of which haploid plants are obtained, have determined many researchers to study the conditions of plants regeneration from ovules and ovaries on a considerable number of plants, among which *Beta* gender.

Baenziger and Schaeffer (1982) have dealt with the study of dihaploid at beet, and Bosemark N.O. (1971) establishes the conditions of obtaining haploids at sugar beet for different genotypes with different degrees of ploid plants which produce seeds. Hosemans and Bossoutrot (1983) (11) initiate gynogenesis in the sugar beet, and Van Geyt and Jacobs (1986) mention the conditions of inducing gynogenesis via callose from ovules and ovaries at sugar beet and the cytoenzymatic modifications that appear during *in vitro* culture.

In this respect, this study presents the conditions of obtaining haploids at the beet through ovules *in vitro* and the perspective of creating dihaploids, taking into consideration the fact that there are many problems in the process of improving this species, problems regarding the obtaining of cytoplasmatic androsterile lines.

## MATERIALS AND METHODS

Diploid and tetraploid genotypes of beet have been used from the collection of The Station of Agricultural Studies Lovrin, Timiş County.

The selected biological material was represented by plants of very good quality, as donators of explants, which were raised in most propitious conditions of greenhouse vegetation, after a period of vernalisation of approximately 90 days, at a temperature of 4°C.

The basis medium Van Geyt and Jacobs (1985) was supplemented with phytohormones according to the experimental variants specified in Table 1. In the first phases of callose induction, active coal was added in a concentration of 0.5% to hold back the inhibitors of gynogenesis. All the categories of used explants were subject to the following experimental treatment:

1. Submergence in ethanol 96% (30 seconds);
2. Washing in hypochlorite of Ca 2% + Tween 40;
3. Washing in solution of $Na_2EDTA$ 5mM (5 minutes);
4. Rinsing with sterilized distilled water three times;
5. Inoculation in the basis medium Van Geyt and Jacobs (1985).

After inoculation the phials were put into the thermostat culture room at a photoperiodical treatment of 18 hours of light (25 μmol $m^{-2}s^{-1}$) – 8 hours of darkness and at a temperature of 18-20° C / 13–15° C.

*Table 1*

The phytohormonal balance used for the induction of gynogenesis to the beet

| Variant | BAP mg/l | AIA mg/l | 2,4-D mg/l | Observations |
|---|---|---|---|---|
| 1. | 0.5 | 0.05 | 0.5 | Callose induction |
| 2. | 0.5 | 0.05 | 0 | Callogenesis |
| 3. | 0 | 0.05 | 0 | Rhizogenesis |

The evolution of ovules growth was verified daily in order to trace out the most propitious moments of subcultures. These appeared after approximately 12–14 days after initiating the culture. The conclusion, as a result of numerous observations, was that the aspect of *comma* of the ovules constitutes the best moment for callose induction ovules at the beet. The spherical ovules have degenerated into culture after approximately 7 days.

## RESULTS AND DISCUSSIONS

### CALLOSE INDUCTION

For organogenous callose induction from ovules the best phytohormonal combination was chosen and only in the conditions of the presence of the ovules next to the ovaries. Thus, the experimental variant ($V_2$) (BAP 0.5 mg/l, AIA 0.05 mg/l and 2,4–D 0.5 mg/l) constituted the starting point for inducing organogenesis from the ovules.

After approximately 12–14 days from inducing the culture, when the ovules that were still found in the ovaries enlarged their volume, a longitudinal incision

was made in order to allow their further evolution. The callose that formed was translucent, slightly opalescent and friable (Fig. 1), characteristic determined by the presence of genes at the level of chromosome 3 (13). Actually, in the experiments of improving the sugar beet in conditions of *in vitro* culture, its growth and development were superior at the addition of this chromosome. *In vitro*, all explants grew in *rosette* and a friable callose was obtained, without any zone of vitrification being formed.

In our preliminary experimental phases, of supervising the way of callose induction from ovules, the ovules were completely detached from the ovary. The results were negative, the ovules necrosed. Van Geyt *et al.* (1988) (18) showed that in *in vitro* culture, the inocula evoluated in a satisfactory way, the plantlets having grown in *rosette*, an aspect determined by the activation of genes at the level of chromosome 4 (13). The same authors mention that the vitrification phenomenon was manifest at 1% of the cases, a reaction which was also possible due to the BAP action and the accumulation of the polyamides (9), factors that confer this undesired aspect to the culture. No callose was formed at the base of these inocula.

The results regarding callus induction show a low rate of callose formation from the ovules, the average being of X = 1.31%. In Table 2 several values are presented regarding the percentage of callose/no. of inoculations, and respectively, the percentage of haploid plants obtained. Thus, there are diploid genotypes which presented an average of callose formation of 1.2%, and others of 2.5% (Intensive II). At the tetraploid forms, the average was higher, of X = 4.62%. Thus, Lovrin 831 M92 had an average of X = 1.1%, Lovrin 919 S92 X = 7.1%, and Tetra Red of X = 16.3%. There are also genotypes whose ovules did not present any reaction to callose induction.

Van Geyt, Speckmann and Jacobs (1986) studied the phenomenon of callose induction from ovules in a comparative way among different varieties of sugar beet and some ancestral species – *B. procumbens* and *B. webbiana* –, but the authors used 0.3 mg/l BAP and specified also the low percent of callose formation and that of plant regeneration.

## THE REGENERATION OF HAPLOID PLANTLETS

The callose obtained from ovules was transferred on a medium of regenerates formed by MB supplemented with BAP 0.5 mg/l, AIA 0.05 mg/l without 2.4-D. After about 8–10 weeks the first haploid plantlets were formed from the ovular callose (Fig. 2). The diploid genotypes presented a low average of regeneration from the callose obtained from ovules (X = 0.42%), and the tetraploid ones had a higher average, X = 2.78% (Table 2).

The haploid plantlets obtained from ovules are of small size and have chlorotic leaves, with an elongated petiole, a character determined by the presence of genes at the level of chromosome 6 (13). At the addition lines, the genes of this chromosome favour the formation of some semierect inflorescences with a reduced growth. Maintained in culture or subcultivated for cloning they have degenerated and finally necrosed. As a result of it in order to obtain viable plants of beet, the primary callose formed by haploid cells was subject to colchicinization, and in the end the haploid cells were created.

*Table 2*

The reaction of diploid and tetraploid beet ovules regarding the formation of the callose and organogenesis on the culture medium Van Geyt and Jacobs supplemented with BAP, AIA and 2.4D

| **The genotype** | **No. of explants** | **% callose from nr. Inoculates** | **% haploid plants** |
|---|---|---|---|
| **Diploid genotypes** | | | |
| 1. Lovrin 189 | 75 | 0.6 | 0 |
| 2. Lovrin 416 / 91 | 110 | 1.2 | 1 |
| 3. Lovrin 861 / 92 | 80 | 2.4 | 0 |
| 4. Lovrin 961 / 92 | 200 | 0.4 | 0 |
| 5. Lovrin 433 / 92 | 125 | 2.1 | 1.2 |
| 6. Gotlob II | 95 | 0 | 0 |
| 7. Timis I | 50 | 1.7 | 0 |
| 8. Cesar | 115 | 0 | 0 |
| 9. Red I | 60 | 2.2 | 0 |
| 10. Intensive II | 100 | 2.5 | 2.0 |
| **Tetraploid genotypes** | | | |
| 1. Lovrin T- 90 | 88 | 3.2 | 1.7 |
| 2. Lovrin 44 R 91 | 121 | 1.2 | 0 |
| 3. Lovrin 450 S 91 | 74 | 0 | 0 |
| 4. Lovrin 977 F 92 | 97 | 2.1 | 2.0 |
| 5. Lovrin 703 G 92 | 160 | 3.1 | 1.3 |
| 6. Lovrin 533 C 90 | 56 | 2.7 | 2.2 |
| 7. Lovrin 831 M 92 | 60 | 1.1 | 0 |
| 8. Lovrin 919 S 92 | 88 | 7.1 | 5.2 |
| 9. Tetra Rosu 33 O | 90 | 9.4 | 7.6 |
| 10. Tetra Rosu 75 C | 110 | 16.3 | 7.8 |

From this dihaploid cellular material, subject to cloning according to the experimental variant $V_2$, dihaploid plants were obtained, which were pure from a genetical point of view, viable and did not present chloritic symptomatology.

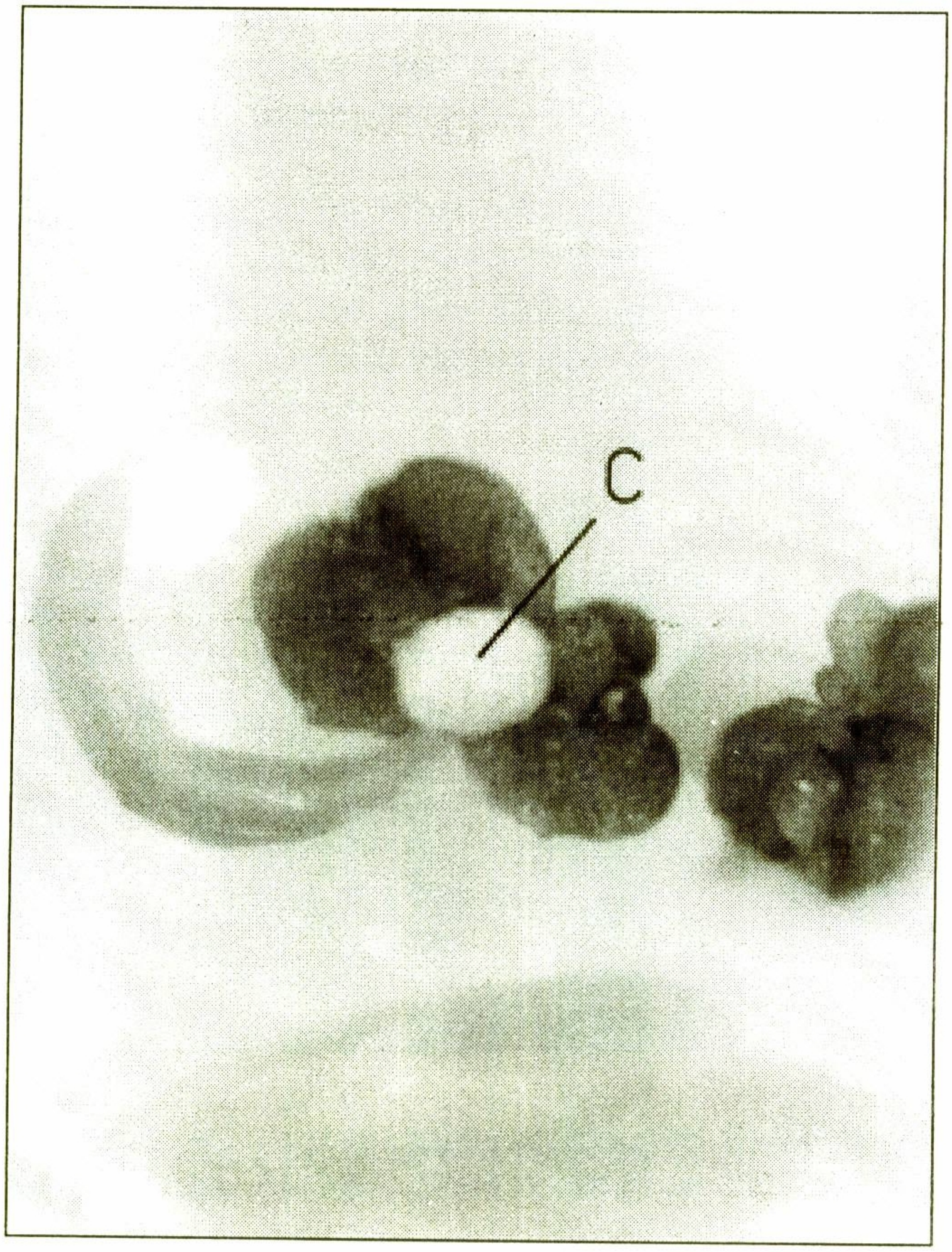


Fig. 1 – Ovular callose at the diploid genotype Intensive II.

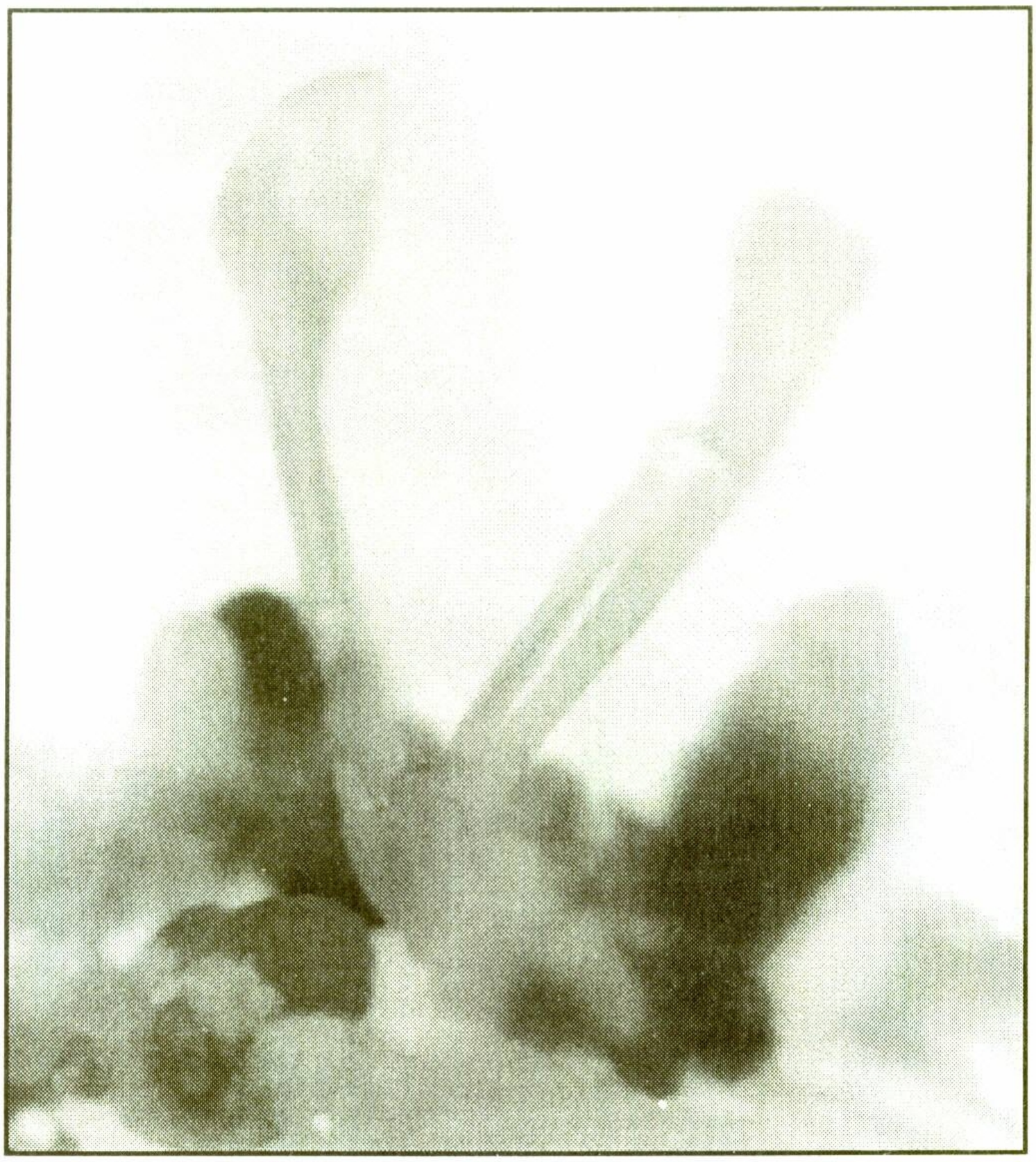

Fig. 2 – Formed haploid plantlets of the diploid genotype Intensive II.

## RHIZOGENESIS

When they reached the size of 2 cm, the plantlets were subcultivated in the medium for the release of rhizogenesis.

It was added only AIA 0.05% for inducing the formation of the roots in the medium destined to this purpose. After approximately 21 days the first filiform radicles were formed, after which the plants were acclimatized and raised in the greenhouse.

It is supposed that the genes of the chromosome 5 would intervene in the rhizogenetic process, but also through the supplementation of the culture medium with BAP. At the addition lines, the genes of the chromosome 5 favour the *in vitro* development, the plants grow in *rosette* and the leaves have a relatively big limb (13).

## CONCLUSIONS

1. For obtaining an organogenous callose from the ovules of beet, it is necessary to use the top of the inflorescence – the first 4–5 mm – which contains a sufficient dose of endogenous hormones in order to release the callose induction.

2. The callose formation is possible only if in the culture medium the coal is added in a concentration of 0.5% and if the ovules are used with a certain morphological configuration, that of *comma*, the spherical ovules are not appropriate for callose induction. It is also necessary that the ovaries be present for the formation of the callose; in their absence, the ovules degenerate shortly after inoculation.

3. The presence of the BAP and AIA phytohormones is necessary in each phenological stage.

4. In order to obtain some viable plants, the haploids were subject to colchicinization; the haploids are chlorotic and present signs of necrosis in subculture.

5. One can conclude from the statistical processing that the genotype has a decisive action on the regeneration of the haploid plants.

6. The haploid and dihaploid plants of the beet, obtained by this procedure, can be further used for other experimental categories of improvement.

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# CYTOGENETIC STUDIES ON SOME RARE AND ENDEMIC SPECIES IN THE ROMANIAN FLORA

ANDREEA CUNIȚĂ*, NICOLETA CONSTANTIN*, GAVRIL NEGREAN**, VERONICA STOIAN*

Our research deals with the genetic characterization of some rare, endemic, threatened and vulnerable plant species. We established chromosome numbers of four species: *Centaurea phrygia* subsp. *ratezatensis* (Prodan) Dostál, *Euphorbia myrsinites* L., *Rumex tuberosus* L. subsp. *tuberosus*, *Tragopogon graminifolius* DC, respectively, 22, 20, 14, 12. These numbers are similar to those in the scientific literature, but as far as we know they are reported for the first time for Romanian populations of these species.

*Key words*: chromosome number, rare and endemic plants.

## INTRODUCTION

Presently, the main trends in all biological sciences are the molecular approach and the search for the genetic background, irrespectively the object of study. This is also valid in the modern taxonomy, where every research involves also genetic characterization of the studied species.

Genetic characterization is even more important for plants than for animals, as the intraspecific and intrapopulational genetic variability is greater. If for animal species chromosome number is constant for every species, plants tolerate not only aneuploid variations, but also polyploids.

In conclusion, we consider the genetic study necessary not only at species level, but also at population level. Our research concerns the cytogenetic characterization of some endemic, rare and/or threatened plant species.

## MATERIAL AND METHODS

The following plant species were characterized:

*Centaurea phrygia* subsp. *ratezatensis* (Prodan) Dostál (*Centaurea ratezatensis* Prodan) (= *Centaurea ratezatensis* Prodan) (12) – Compositae, subgenus Jacea, section Lepteranthus (16). Prodan described this perennial plant in the Țarcu-Petreanu Mountains (2, 12). He erroneously named this species "ratezatensis", as he thought that the collecting place is in the Retezat Mountains. This error is also found in "The Romanian Flora" (14) and "Flora Europaea" (16). This species is cited in the red list of higher plants of Romania (11) as rare and endemic. *Puccinia calcitrapae* DC. (BUCM 67.272, 129.453, 129.604, 129.685, 135.402, 135.413), a rust, frequently parasitizes this species.

The rust is sometimes parasitized by a hyperparasite: *Eudarluca caricis* (Fr.) O. Eriksson (BUCM 135.403). *Centaurea phrygia* ssp. *ratezatensis* is "matrix nova" for both fungi. The seeds were collected in Țarcu-Petreanu Mountains, Valea Netiş, (45°21'42"N, 22°44'32"E, at 925 m altitude (BUCM 135.413)); the plant was collected in Valea Mare, in the same mountains, also (45°23'35"N, 22°46'03"E, at 800 m altitude (BUCM 129.604)).

*Euphorbia myrsinites* L. – Euphorbiaceae, section Myrsiniteae (16). Perennial, sempervirescent species of Mediterranean origin. It is very rare in Romania and is present on the red list of higher plants in Romania (11) as vulnerable and rare. It is found in the southern and western part of the country - the Danube Defile – and in the Northern Dobrogea (14). Corresponding to "Flora Europaea" (16), in some Mediterranean islands three species were described, without specifying the present rank. Also, based on the cyathium glands colour (red), a subspecies was described in Italy. Our material has these glands red, but we do not consider it to deserve the subspecies rank. We collected the material at west of Agighiol, on Dealul Pietros (45°01'41"N, 28°51'50"E, at 130 m altitude, on 1997.05.14. (Herb. BUCM 134.620)).

*Rumex tuberosus* L. subsp. *tuberosus* – Polygonaceae, subgenus Acetosa (16). It is a Mediterranean species and it has its areal at the northern limit of our country. It is cited in the red list (8, 11) as rare and vulnerable. Beside the places indicated in "The Romanian Flora" (14) we add Danube Delta, Letea Forest (leg. G. Negrean), Hagieni Forest (leg. G. Negrean, 28.05.1971 (BUCM 40.284)), south-west of Mircea-Voda railway station (leg. G. Negrean, 29.05.1980 (BUCM 55.964)), Mangalia (leg. G. Negrean, 12.05.1977 (BUCM 50.410)), Eforie Sud (leg. G. Negrean, 26.06.1998 (BUCM)). The seeds were collected in Hagieni Forest (43°49'11"N, 28°27'25"E, at 10 m altitude, 3.07.1998).

*Tragopogon graminifolius* DC. – Compositae (16). It is or rare or endemic, neither vulnerable nor threatened, but it is a new species. We consider of interest to take this species into consideration. Dihoru, 1998 (7) published it as new for Romania. It is found often enough in the western part of Bucharest (in the neighbourhood of Interpol Antenna, near Ciurel Ştrand, in front of the Institute of Biology of Romanian Academy, at Grozăveşti, on the banks of Dâmbovița, in front of the greenhouse of the Botanical Gardens). Sometimes it appears and disappears suddenly. It is of Caucasian origin, adventive in Romania. Our material was collected in Grozăveşti area (44°26'15"N, 26°03'36"E, 75 m altitude), in July 1998.

For all the studied species, the seeds were germinated in semisterile conditions, on moisten filter paper, prefixed in 0.05% colchicine (when roots were 0.5–1 cm long), fixed and preserved in acetic ethanol. Chromosomes were studied through Feulgen method. Metaphasic plates were analyzed in light microscopy (Amplival microscope) and photographed with 100 x objective, with an Exacta-Varex camera.

## RESULTS

Analyzing minimum 10 metaphases per species, we observed:

*Centaurea phrygia* subsp. *ratezatensis* chromosome complement comprises 22 metacentric and submetacentric chromosomes, in somatic cells (Fig. 1);

Fig. 1 – *Centaurea phrygia* subsp. *ratezatensis* mitotic metaphase.

Fig. 2 – *Euphorbia myrsinites* mitotic metaphase.

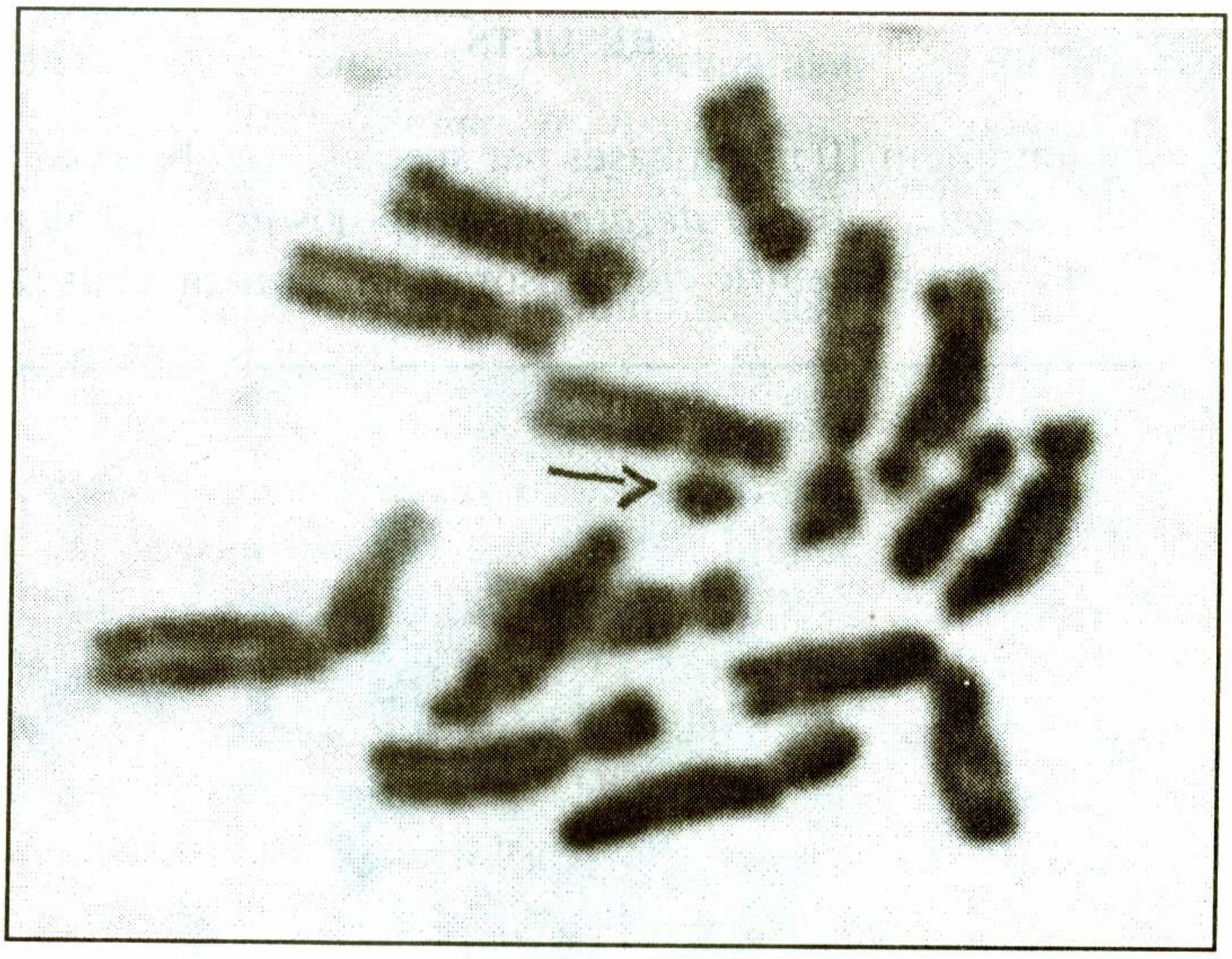

Fig. 3a – *Rumex tuberosus* subsp. *tuberosus* mitotic metaphase.

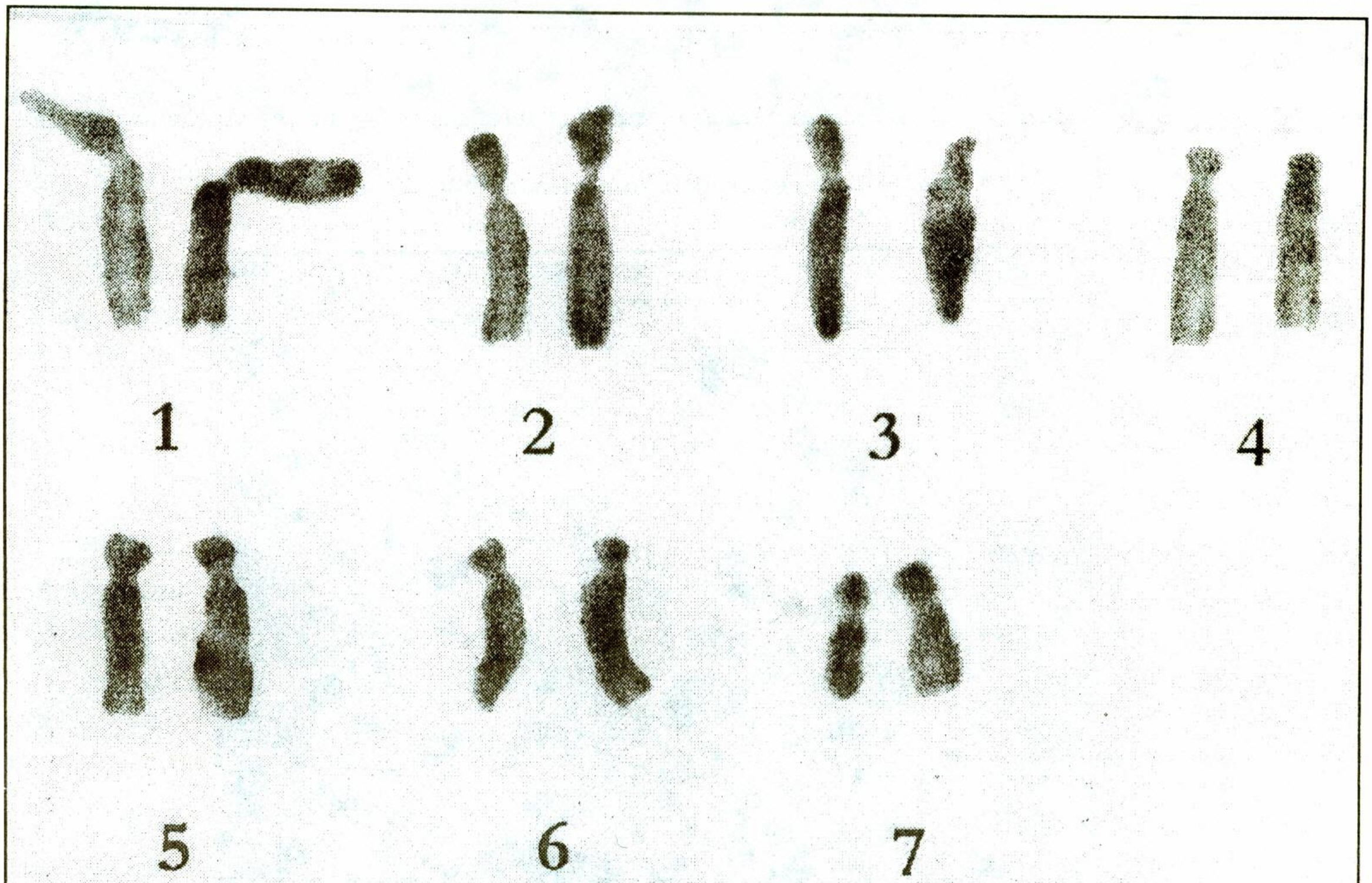


Fig. 3b – *Rumex tuberosus* subsp. *tuberosus* karyotype.

Fig. 4a – *Tragopogon graminifolius* mitotic metaphase.

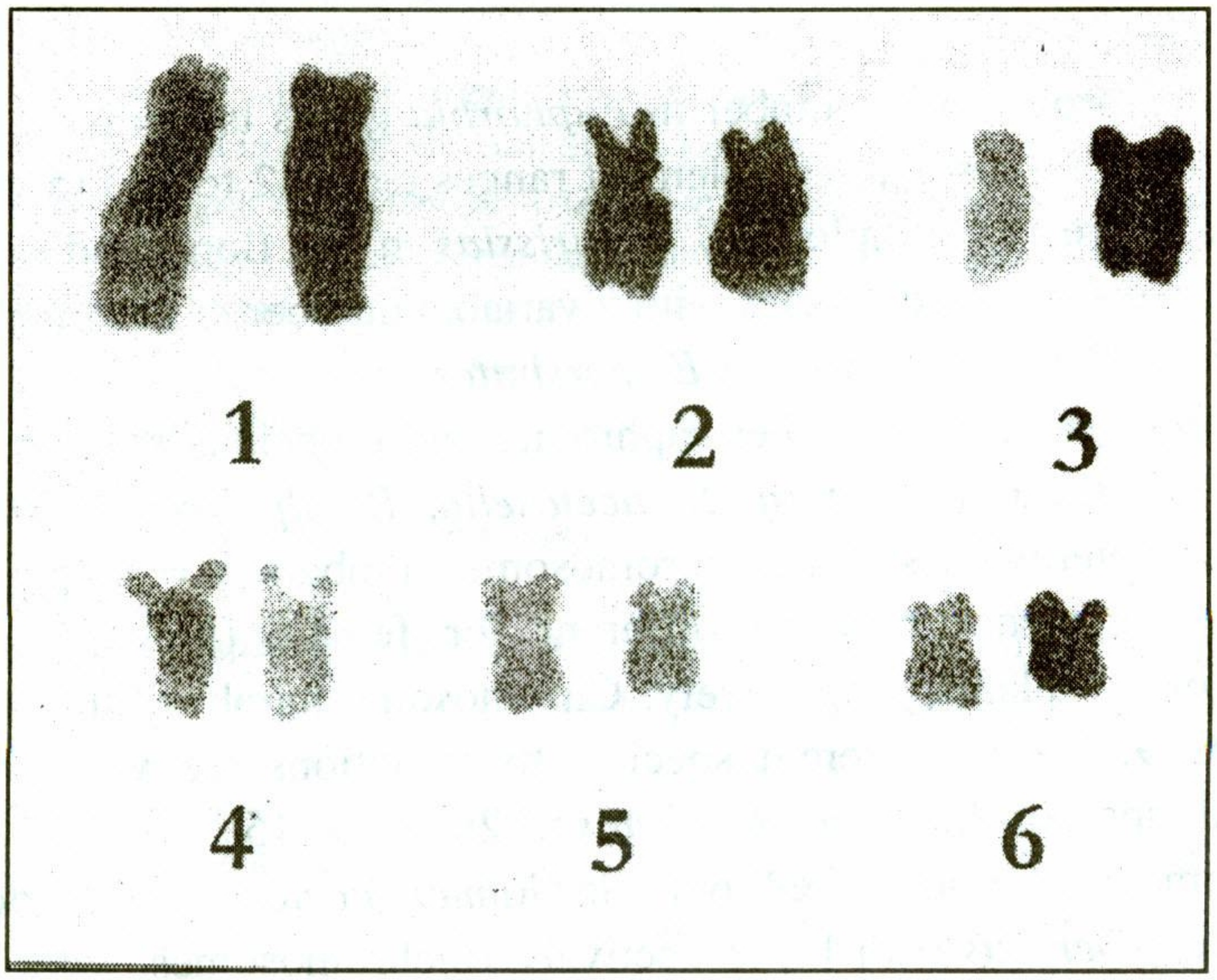


Fig. 4b – *Tragopogon graminifolius* karyotype.

In *Euphorbia myrsinites* somatic cells, we found 10 pairs of metacentric and submetacentric chromosomes (Fig. 2);

In *Rumex tuberosus* subsp. *tuberosus* meristematic cells are present 14 chromosomes – a large metacentric pair (no.1 in the karyotype), 6 smaller submetacentric pairs (nos. 2–7), and an accessory chromosome (Fig. 3);

In *Tragopogon graminifolius* we found 12 chromosomes, from which pairs no. 1–4 are submetacentric (pairs no. 1 and 4 bearing satellites) and pairs no. 5 and 6 – metacentric (Fig. 4).

## DISCUSSIONS

Unlike cultivated species, for which the number of cytogenetic studies is huge, wild, spontaneous species, which are not of practical interest, are little studied.

In *Centaurea* genus, the basic number of chromosomes x = 5, 6, 7, 8, 9, 10, 11, 12, 13, and the complement of different species varies correspondingly (5, 6, 9, 10). There are species with different levels of ploidy (*C. triumfetti* – diploid and tetraploid) and also there are chromosomes B in variable numbers (9). For *C. phrygia* species, 2n was determined as 22 (5, 6, 9), but the literature does not offer data for each subspecies. Our studies confirm this number for subspecies *ratezatensis*.

Basic chromosome number in *Euphorbia* genus is x = 6, 7, 8, 9, 10 and the somatic cell chromosomal complement ranges from 12 to 200 (5, 6, 9, 10). Within the genus, there are tetraploid (*E. cyparissias* in our flora) and octoploid species; also there were reported species with a variable number of chromosomes (6, 9, 10). We confirm 20 chromosomes for *E. myrsinites*.

*Rumex* genus includes hermaphrodite and dioecious species; among the last ones, in our flora *R. acetosa, R. acetosella, R. alpestris, R. thyrsiflorus* were discovered heterosomes. Basic chromosome numbers x = 4, 5, 7, 8, 9, 10; the somatic complement may be different for female (homogametic) and male (heterogametic) plants, respectively. Chromosome number fluctuates from 8/9 to 200 (5, 6, 9, 10) in different species. Its variations are very important within individual species (i.e. for *R. acetosa*: 2n = 14/15, 21, 22, 29). Accessory chromosomes were identified only in *Rumex acetosa* (9). Fedorov, 1969 (9) reported *R. tuberosus* with 14, respectively 16 chromosomes, without heterosomes. Sex determination is probably genic. Cytogenetic study of tuberosus subspecies showed a complement constituted of 14 chromosomes for our population.

The species belonging to *Tragopogon* genus have x = 6, 7, 9, and the variability of chromosome number is very small, opposite to previous species: 12, 14, 24 (6, 9, 10). Polyploidy (tetraploidy) is certain only for *T. cupanii; T. reticulatus* was reported with a variable chromosome number in different populations, but none of these species is present in our flora. *T. graminifolius* is reported with 12 chromosomes, and we confirm this number.

In conclusion, our study confirms and completes the literature; at the same time, it establishes the chromosome numbers for the peculiar situation of these species populations in our country*.

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# CONTRIBUTIONS TO THE HISTO-ANATOMICAL STUDY OF SOME *ACHILLEA* L. SPECIES FROM THE ROMANIAN FLORA

RODICA RUGINĂ, CONSTANTIN TOMA

Notre étude vise quatre espèces de plantes appartenant au genre *Achillea* (*A. distants* W. et K. ssp. *stricta* (Schleich. et Koch) Jauch., *A. nobilis* L. ssp. *neilreichii* (A. Kern) Form., *A. ptarmica* L., *A. schurii* Sch. Bip). Sont analysés du point du vue histo-anatomique la tige, la feuille et les folioles involucrales. On met en relief des caractères structuraux avec valeur de diagnose.

*Mots clefs: Achillea*, anatomie.

## INTRODUCTION

The genus *Achillea* is represented in Romanian flora by 28 species and 18 hybrids, which are spreading from the plain to the mountain region (3).

This genus is one of the most genera from our flora with difficulties in the determination of species on the basis of morphological features. That is why the present contribution has the main aim to present a histo-anatomical study which spots lights on the structural characteristics with diagnosis value.

Moreover, the histo-anatomical examination is motivated by the fact that species of genus *Achillea* have great therapeutical utilizations. Or, for the precise determination of drugs, in the event of dry and incomplete material, microscopic observations are necessary, thus avoiding the frequent impurifications of these ones (4,5).

The emphasis on the structure characteristics with diagnosis value is one of the constant preoccupations of the Department of plant morphology and anatomy from Jassy. In the latest decades there have been analyzed species of many genera of gymnosperms and angiosperms flora, such as: *Berberis, Cotoneaster, Fraxinus, Iris, Lathyrus, Lychnis, Lycopus, Plantago, Potentilla, Salix, Salvia, Stachys, Syringa, Vicia,* a.s.o.

Our researches were focused on the species with therapeutical value (6), finalizing in the handbook "*Anatomy of medicinal plants*" (7), in which there were analysed 128 indigenous and exotic taxons, the results being illustrated by 1154 figures.

Some structural characteristics concerning species of *Achillea* are concisely presented in the work synthesis "*Anatomy of the Dicotyledons*" (published by Metcalfe and Chalk (1), and "*Anatomy des Blattes*" by Napp-Zinn (2).

The material studied in the present paper, collected in the flowering stage, proceeds from the collections of the Botanical Gardens of Jassy. (*A. distans* W. et K ssp. *stricta* (Schleich. et Koch) Jauch., *A. nobilis* L. ssp. *neilreichii* (A. Kern) Form., *A. ptarmica* L.) and the Ceahlău Massif (*A. schurii* Sch. Bip.).

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The processing of the examined material was done in accordance with the method published in our previous papers too (6, 7). The structure of aerial vegetative organs (stem, leaf) and involucral folioles was analyzed.

## RESULTS

### *The aerial stem*

In transverse section at the middle level, it presents a circular contour, modified by a variable number of ribs (7–8 in *A. nobilis*; 10–11 in *A. distans*, or more in other species).

The epidermis has isodiametrical cells either everywhere or only in ribs (*A. distans*). The stomata are very rare (*A. ptarmica*), or frequent (*A. distans, A. nobilis*). The uniseriated (4–5 cells) a trichomes (tectory hairs) have short basal cells (usually 2) with suberified walls and long flexuous terminal cell, only in *A. nobilis* the comparatively larger basal cells have nonsuberified walls. The secretory hairs have bicellular stalks, and sphaerical, elliptical tetra- or octo-cellular glands; they are absent in *A. ptarmica*, rare in *A. distans, A. nobilis* and frequent in *A. schurii*.

The cortex is chlorenchymatous, of variable thickness with 6–8 layers (*A. distans, A. nobilis, A. schurii*) or 9–10 layers (*A. ptarmica*). The last mentioned species has numerous intercellular spaces while *A. distans* and *A. nobilis* have two cortical bundles.

An angular collenchyma is usually developed in ribs while inside, near the vascular bundles, one can notice 1–2 secretory canals, a zone in which the casparian-type endodermis is also visible.

The vascular tissues are of fascicular type, the numerous bundles (26–28 in *A. distans*, 29–30 in *A. ptarmica*; 30–32 in *A. nobilis*) being situated after a sinuous ring.

The phloem is made of sieve tubes, companion cells and a few elements of parenchyma but also of fibres, more numerous in *A. nobilis, A. ptarmica*.

The xylemic vessels, seriated only, sticked in a lignified or nonlignified (*A. schurii*) ligneous parenchyma are narrow (10–20 μm) in *A. distans, A. ptarmica, A. schurii*) or wide (45–50 μm) in *A. nobilis*.

All bundles have an outer sheat, therefore in pericycle position, made up of sclerified and lignified fibres.

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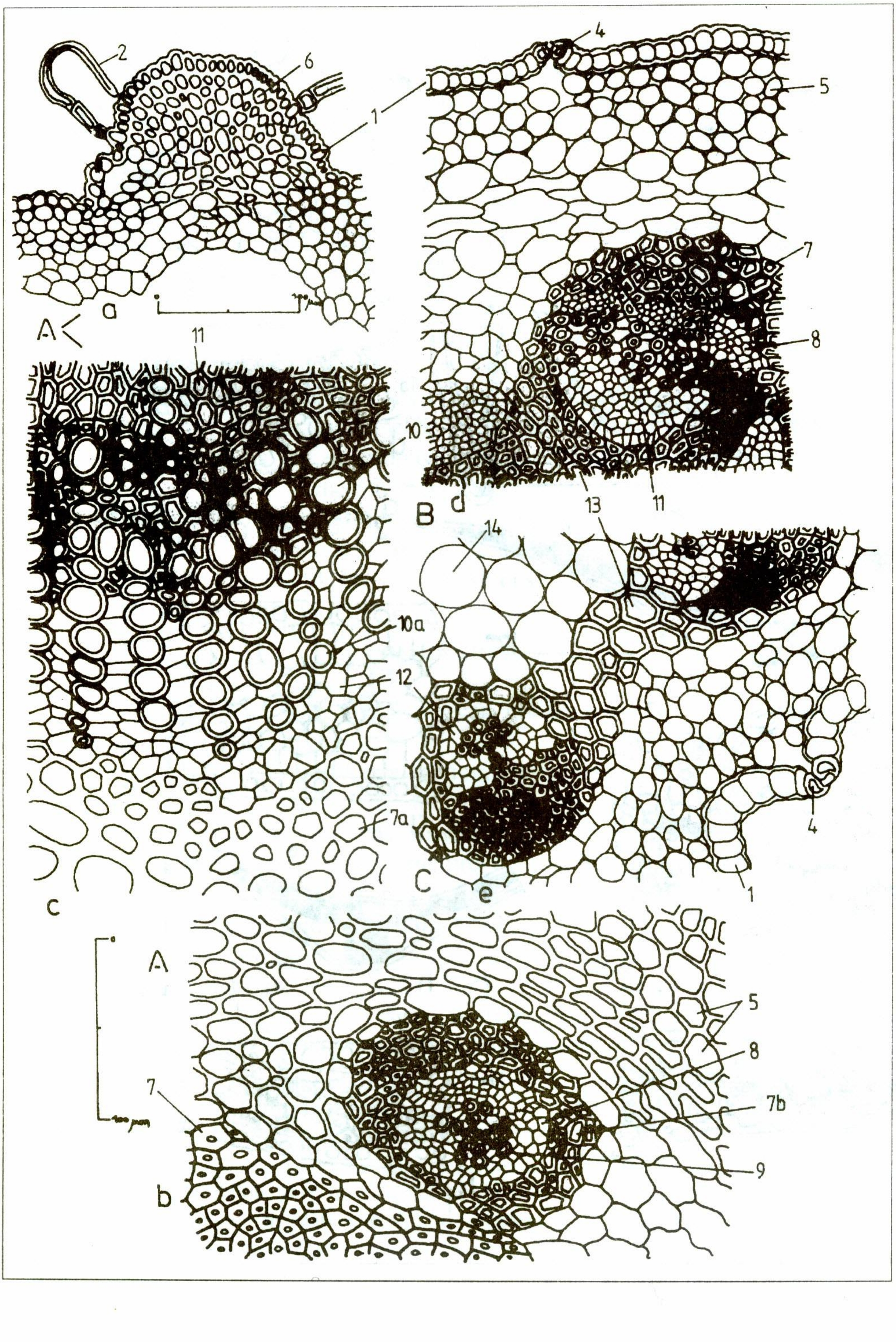
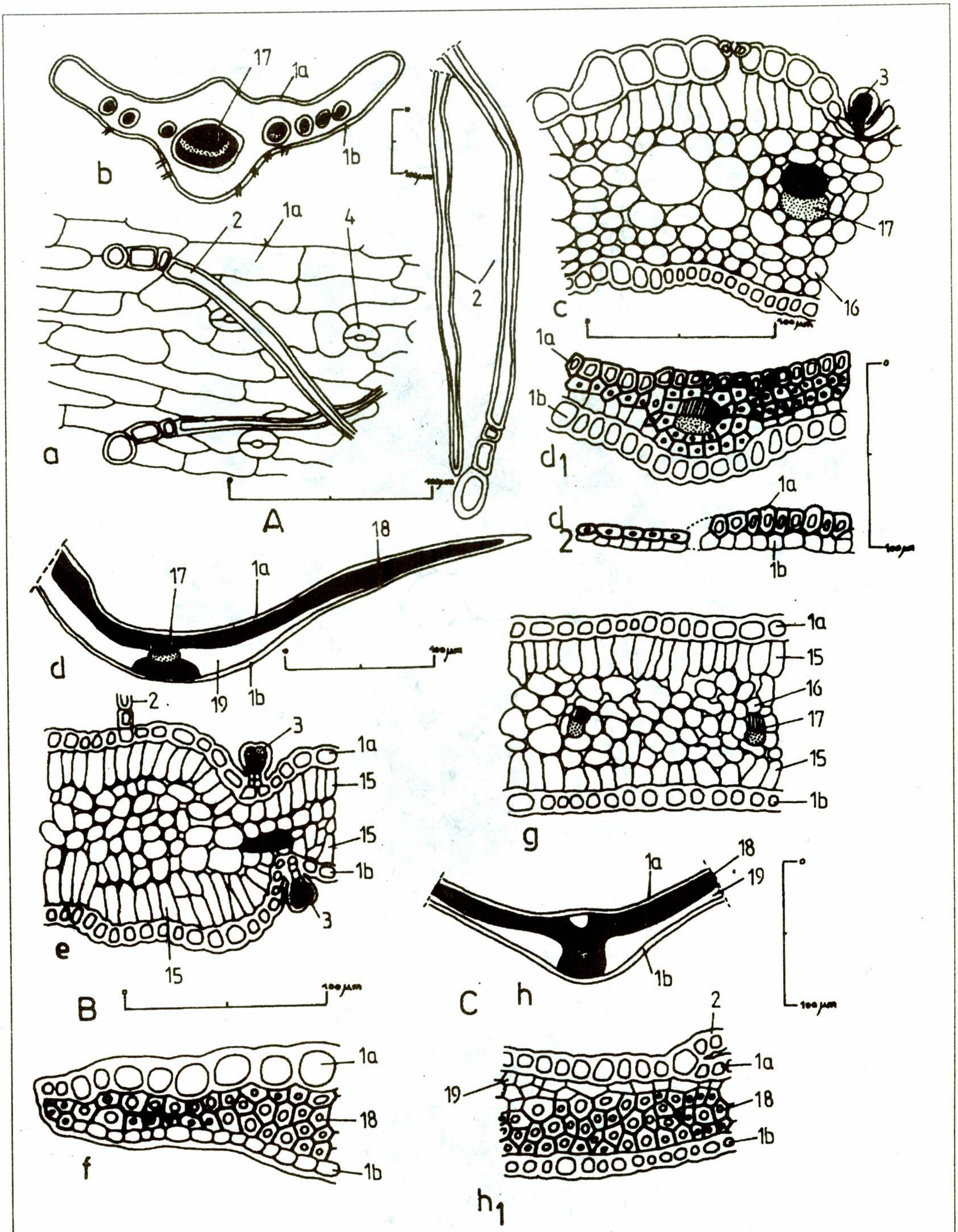
**Plate I** – *Achillea distans* ssp. *stricta* (A); *Achillea ptarmica* (B) *A. schurii* (C). Structure of the stem. *a-c* details of epidermis and cortex of one rib level (*a*), of internal cortex with a concentric bundle (*b*), and internal xylem (*c*); *d*. detail of epidermis, cortex and a vascular tissue; *e* – the same and in addition to the medullo (pith).

# Plate I



## Plate II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1b
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1a
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1b
C
h
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1a
19
18
1b
h1
1a
18
1b
f

*The pith*

Is either parenchymatous cellulosic or easily sclerified (*A. distans*, *A. nobilis*) partially replaced by an aeriferous cavity (*A. ptarmica, A. schurii*).

*The leaf*

In surface view, the epidermis has irregular polygonal (*A. nobilis, A. schurii*) or rectangular-elongated cells (*A. distans, A. ptarmica)* along the lacinia with rare and superficially waved lateral walls (*A. distans, A. nobilis, A. ptarmica*) or deep and frequently waved ones (*A. schurii*).

In transverse section the lacinia has a variable shape function of the species and the sectioned level, being wide (*A. ptarmica*) and narrow banded or arc-banded (*A. distans, A. nobilis, A. schurii*) with a slight thickening just near the midvein.

All the analysed species have an amphistomatic limb with a bifacial-equifacial structure; only *A. schurii* has a bifacial – dorsiventral structure.

*The involucral folioles* are lamellar-arched in transverse section. The internal ones have a homogeneous – type mesophyll (3–4 layers) while the external ones have a different structure function of the thickness and position of sclerenchymatous lamella in the mesophyll. This is thick (3-4 layers) in *A. ptarmica*, or thin (1–2 layers) in *A. distans*, *A. nobilis, A schurii*, with subepidermal, adaxial (*A. distans, A. nobilis*), abaxial (*A. schurii*) or central (*A. ptarmica*) positions.

## CONCLUSIONS

From the above investigations one can notice a series of histo-anatomical characteristics with diagnosis value:

- at the *stem* its contour in transverse section, the epidermis structure (cells shape, types of stomata and hairs), the cortex structure (number of layers, collenchyma and chlorenchyma structure), presence or absence of cortical bundles and secretory canals; structure type of vascular tissue, number of bundles, shape and size of ligneous vessels, their arrangement;
- at the *leaf*, the shape of epidermal cells, in surface view, the presence or absence of stomata at the two faces, or the trichomes and secretory hairs, their structure, the structure type of the mesophyll;
- at the *involucral folioles*, their shape in transverse section, the position and development degree of the sclerenchymatous lamella.

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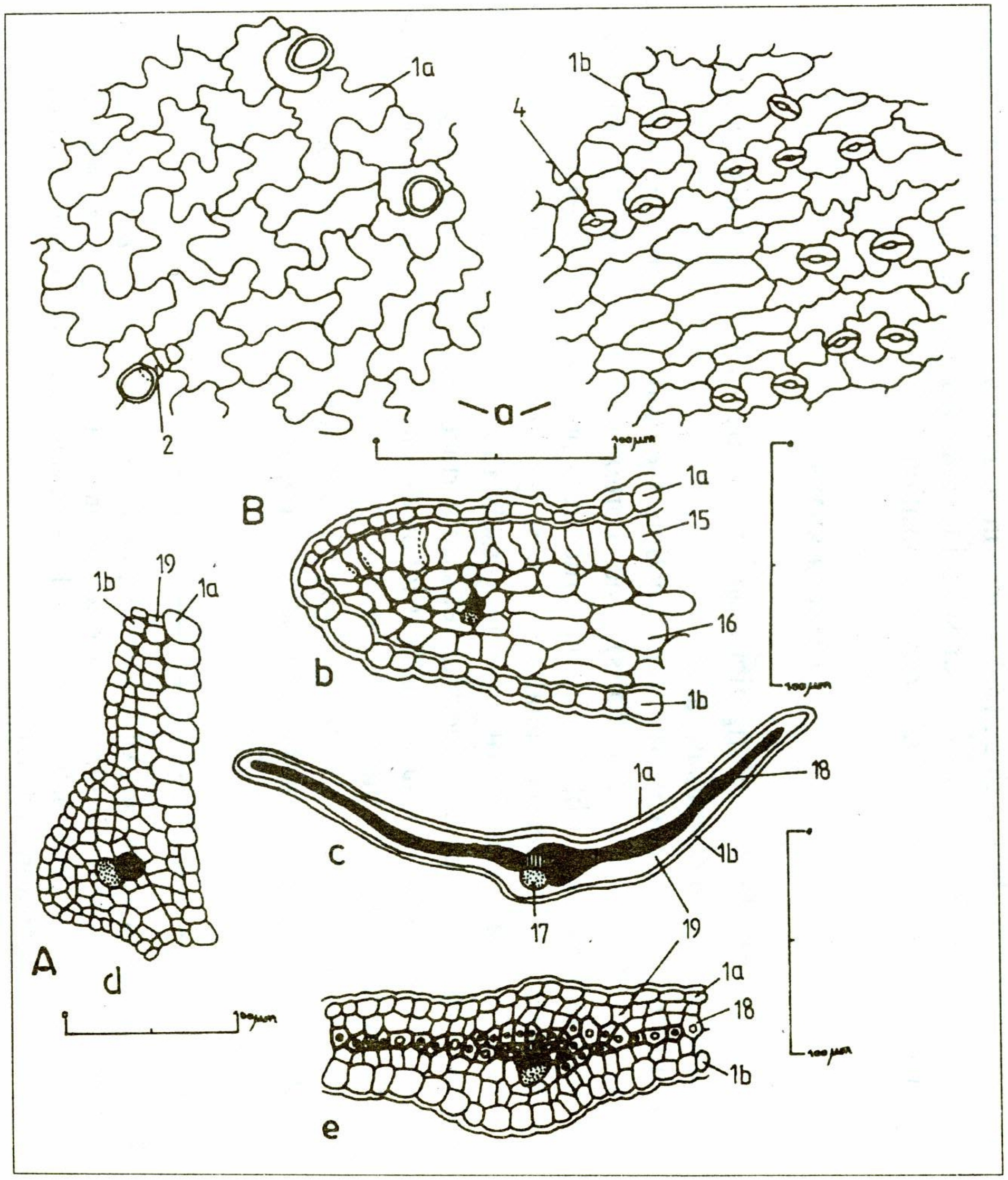
**Plate II** – *A. distans* ssp. *stricta* (A); A. *nobilis* ssp. *neilreichii* (B); A. *ptarmica* (C). Structure of leaf (a-c) and of involucral foliole (d, f, h) a – epidermis in surface view; b, c, e, g - diagram (a) and details (c, e, g) of leaf pinule in transection; d, f, h – diagrams (d, h) and details of involucral foliole of middle ($d_1$, $d_2$) and margin ($d_2$, f) part.




## Plate III

*A. ptarmica* (A); *A. schurii* (B). Structure of leaf (a, b) and involucral foliole (c-e). a – epidermis in surface view; b – detail of leaf pinule in transection of margin part; c – e – diagram (c) and details of internal (d) and external(e) involucral folioles. 1 – epidermis (1a – upper, 1 b – lower); 2 – trichomes; 3 – secretory hairs; 4 – stomata; 5 – cortical parenchyma; 6 – collenchyma; 7 – sclerenchymatous fibers (pericyclic), 7 a – the same, perimedullary, 7 b – the same, perifasciculary, 8 – phloem; 9 – xylem; 10 – metaxylemic vessels; 10 a – protoxylemic vessels, 11 – xylemic lignified parenchyma; 12 – xylemic cellulosic parenchyma; 13 – medullary ray; 14 – medulla; 15 – palisadic tissue; 16 – spongy tissue; 17 – vascular bundle; 18 – sclerenchymatous sheath; 19 – cellulosic mesophyll.

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# MORPHO-ANATOMICAL CHARACTERIZATION OF THE ENDEMIC SPECIES *ANDRYALA LEVITOMENTOSA* (E. NYÁR.) SELL.

ANCA AIFTIMIE-PĂUNESCU, CONSTANTIN TOMA

In this paper it is analyzed the structure of vegetative organs of the endemic species *Andryala levitomentosa* for the first time. Our data confirm that this species is a relict one and offer more arguments for the affiliation on *Andryala* genus, but also contradict the previous descriptions concerning the presence of thick and ligneous roots.

*Key words*: morpho-anatomical, endemic species, *Andryala levitomentosa.*

## INTRODUCTION

According to the flora and vegetation studies, in European flora there are just 5 species of *Andryala* genus: *A.agardhii, A.laxiflora, A.levitomentosa, A.integrifolia* and *A.ragusina*. Among these, *A.levitomentosa* is the only one belonging to this genus in the Romanian flora. It was quite recently discovered (Nyárády,1961) and accepted as a Romanian phytoendemit.

This plant is considered one of the rarest and strangest species in our country. It is found only on "Pietrosul Broştenilor" Mountain on "Pietrosul Bogolin" summit being the only member of this genus which grows at such a high Northern latitude (47°), like the other species belonging to Mediterranean zone (42°).

This relict plant makes botanists raise a lot of questions about its restrictive ecology and peculiarities of multiplication (merely on a vegetative basis).

As it results from the synthesis works upon the Dicotyledons (Metcalfe and Chalk, 1972) or generally the Angiosperms (Napp-Zinn, 1973, 1974) from different handbooks (Deysson, 1965, Esau, 1965) there are a lot of papers on the anatomy of plants belonging to the *Asteraceae* family. We have studied ourselves some species from this family in some papers on the anatomy of medicinal plants (Rugină and Toma, 1989, Toma and Rugină, 1998).

## MATERIALS AND METHODS

The samples were collected from "Pietrosul Broştenilor" Mountain, 1700 m high. At the time of collection we appreciate that there are almost 200 plants.

The fixing, coloring and analyzing methods are the customary ones made in the Anatomical Laboratory of the University of Iaşi. The histo-anatomical analysis was made on permanent preparations which comprised superficial sections of the leaf epidermis, longitudinal sections through the rhizome, cross sections through the root, the stem (rhizome and aerial stem) and the leaf (ocrea, petiole and lamina).

The reading of all preparations was made at Novex-K microscope, the drawings were made at MC1 microscope and the epidermis photos at electron-scan microscope and those from the stem at Novex-K microscope.

## RESULTS

**The cross-sectioned root** (Plate I, fig. 1, Photos 4, 5) has a circular outline. Rhizodermis with absorbent hairs is early exfoliated; a zone of suberose cells, two- or three-layered, forms between this and cortical parenchyma. The bark is thick, made of three different zones: exodermis (two-layered, with bigger and suberificated cells), cortical parenchyma (5–6 layers of cells tangentially elongated, slightly disorganized forming air cavities with irregular outlines) and endodermis (with small cells with thickened walls of Casparyan type).

The central cylinder is slightly thick having a three-arch structure. At the internal face of the phloemic bundles there are cambial arches, which produce some secondary xylemic vessels with a large diameter. The metaxylem vessels occupy the central part.

The root is strongly flat on the lower part where the structure is typically secondary. There are two distinct peridermis being separated by cellulosic parenchyma with suberificated and radially disposed cells as a result of phellogen activity. Into the parenchyma situated between the peridermis there are several air cavities of irregular outline.

The central cylinder has an asymmetric structure; the phloem is in the external part and the xylem on the internal one, all covered by the internal suberous zone (internal periderm); this is concave on a side of the root and between this and the external periderm there is xylemic tissue, which is an abnormal structure.

In the phloemic tissue there are alternative tangentially discontinuous zones of phloemic elements and laticiferous elements with zones of cellulosic parenchyma. The xylemic tissue has vessels with variable diameter randomly disposed in the cellulosic parenchyma. Although the root structure is of secondary type with a discontinuous cambial zone, but thick between the phloem and the xylem, the xylemic fibers are missing. The secondary xylem has the same structure as in the previous description, between the two periderms.

**The stem** (Plate I, fig. 2–4; Plate II, fig. 1–5, Photos 6, 7) is made of subterranean zone (thick and lignous, confused in the previous botanical papers with the root) and an aerial part (made by scape and a floriferous stem).

The subterranean zone of the stem (rhizome) has also a secondary structure. The primary bark is almost exfoliated, at the edge of the organ being a periderm with suber and collenchymatised phelloderm.

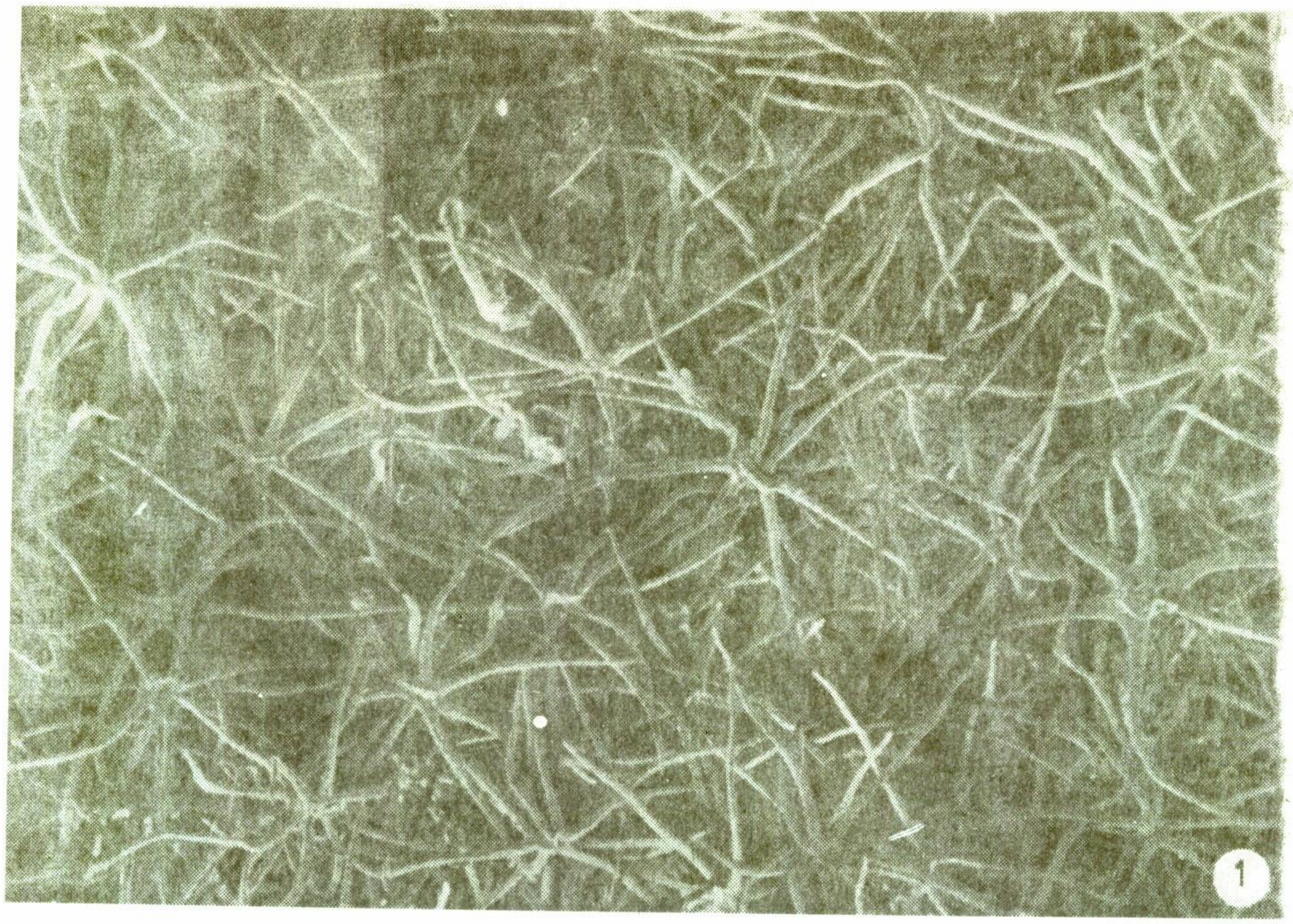



Photo 1 – General view of hairs.

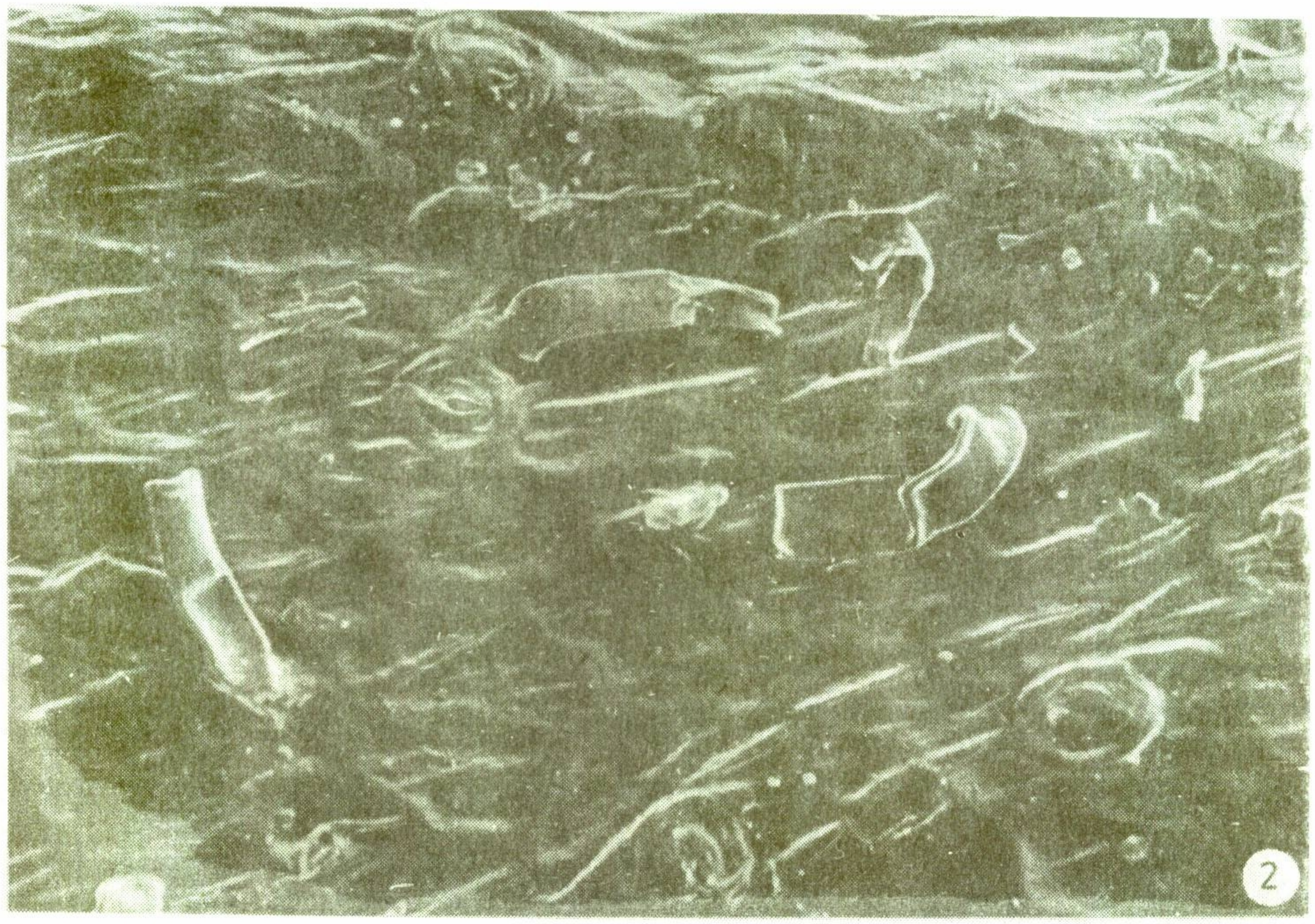



Photo 2 – General view of stomata.

**Photos 1, 2, 3 – Electron-scan microscopical view of the epidermis.**

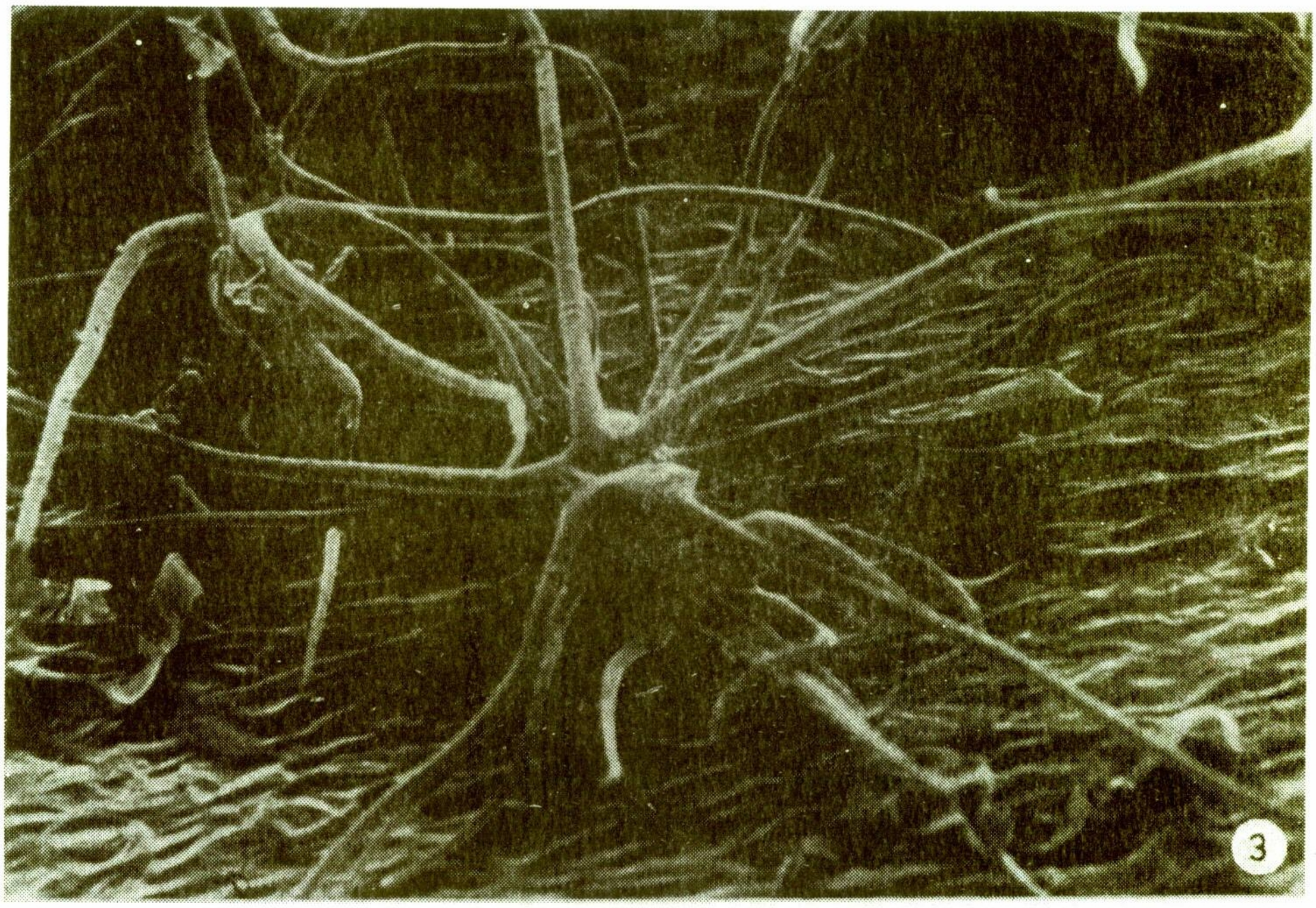



Photo 3 – Detail of a star-shaped covering hair.

Photo 4 – Microscopical view from cross-section of root with secondary structure.

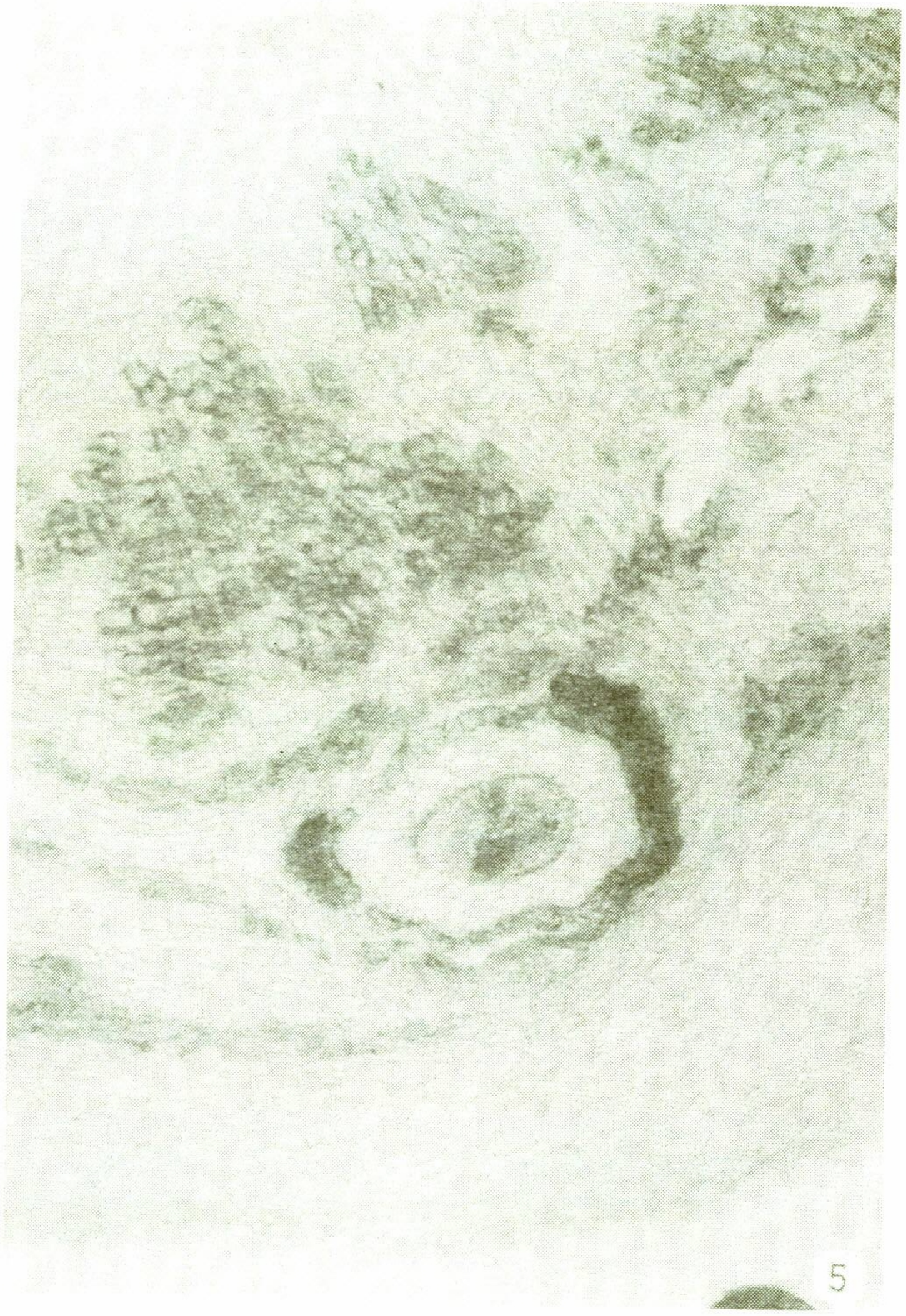



Photo 5 – Microscopical view from rhizome with root in cortical parenchyma.

Photo 6 – Microscopical view from cross-section of scape.

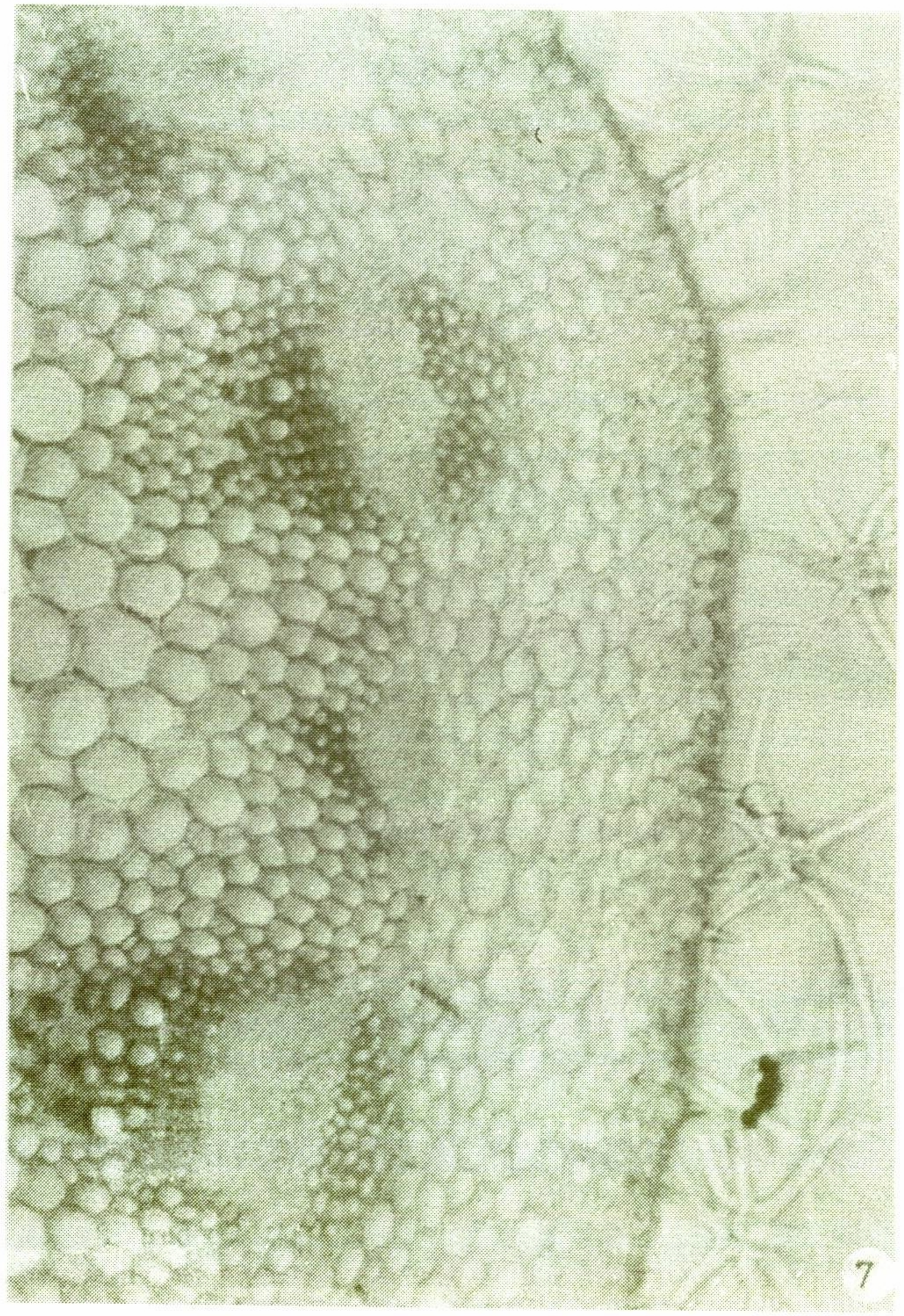


Photo 7 – Microscopical view from cross-section of floriferous stem.

The central cylinder is very thick with vascular bundles of different size separated by medullar rays. In phloem there are alternative tangentially discontinuous zones of phloemic elements and laticiferous elements with zones of cellulosic parenchyma.

On the internal part of the xylemic tissue there is a continuous zone of suberificated cells, similar to a normal periderm from the edge of rhizome.

From place to place there are forming adventitious roots and numerous air cavities (in primary bark, medullar rays, xylem and pith), with variable size and outline.

The pith is thick parenchymatous of meatus type.

In the phloemic tissue and in the primary bark, there are cross-sectioned adventitious roots, with a two-arch structure.

The aerial stem, at the basal internode has an irregular circular outline in cross-section.

The epidermis has isodiametric cells with thickened and cutinized walls. Most of the epidermal cells are transformed into covering pluricellular hairs. On the upper part there are 1–2 levels of monocellular branches that have star-shape in front view, each with 5–7 branches with very thick walls. The pedicel of each hair has cells with very thin and cellulosic walls.

In hypodermic position there is a zone of suber with variable thickness on the organ size.

The primary cortical parenchyma is thick and of meatus type.

The conducting bundles are of primary type structure being separated by parenchymatous cellulosic medulary rays of variable dimensions.

The phloem has the same size like the xylem and has also alternative discontinuous tangentially zones of phloemic elements and laticiferous elements with zones of cellulosic parenchyma. The xylemic tissue has vessels with different diameters irregularly disposed on the cellulosic parenchyma. The meristematic tissue between the phloem and xylem is many-layered and sometimes is present between the vascular bundles resembling a cambium. But even if this meristimatic tissue starts working it produces only vessels and parenchyma of xylem, on the internal part, but never fibers (the same situation in the secondary structure of the root).

On the terminal internodė level (floriferous) which has a circular outline in cross-section, the structure is typically primary. Epidermis has cells with internal and external wall more thickened than the others. A fine-streaked cuticle covers the external wall. At this level there are the same covering star-shaped hairs, but less frequent. From place to place they are also very rare covering monocellular hairs. We have also observed glandular hairs with the pedicel made by two parallel series of cells (19-12) and a pluricellular gland made by 6–10 cells.

The bark, in hypodermic position, is slightly collenchymatised, in front of the small prominent ribs, but for the rest it is parenchymatous cellulosic of meatus type.

Phloemic and xylemic of amphicribal type vascular bundles are also in the cortical parenchyma.

Arranged on a circle the big bundles and the small ones alternate in the central cylinder. Rare elements of laticifers and a zone of mechanic elements can be observed on the edge of the phloem from the big bundles. The phloem is made of sieve tubes and auxiliary cells and the xylem has radial vessels, separated by cellulosic parenchyma.

Medullary rays of different size have cells with less thickened walls, especially in front of the xylemic tissue.

The pith is thick of parenchymatous-cellulosic type, in central position forming an air cavity.

**The leaf** (Plate III. fig. 1–6, Photos 1–3) comprises three distinct parts: ocrea, petiole and lamina.

The ocrea has in cross-section the shape of the "V" letter with divergent branches. The midvein is prominent in the lower part. The ocrea is surrounded by a thick tomentum made by star-shape covering hairs (alike those from the stem level, but more frequent). The epidermis has isodiametric cells with internal and external wall thicker than the others. The fundamental parenchyma is a little collenchymatised in hypodermic position. A lot of leading bundles (5–7) of collateral type with decreasing size on the edge of ocrea are observed in the fundamental parenchyma. All bundles have primary type structure like the one of the stem and there are laticiferous elements on the peripheral zone of the phloem.

The petiole has in cross-section a semicircular outline altered by two latero-adaxial ribs. The structure is similar to that of the stem. The petiole is completely covered by a thick tomentum made by star-shaped covering hairs.

Elements of collenchyma can be observed on the edge of bundles and also on the latero-adaxial ribs.

The laticiferous elements are present with the same frequency as on the ocrea, on the edge of phloem.

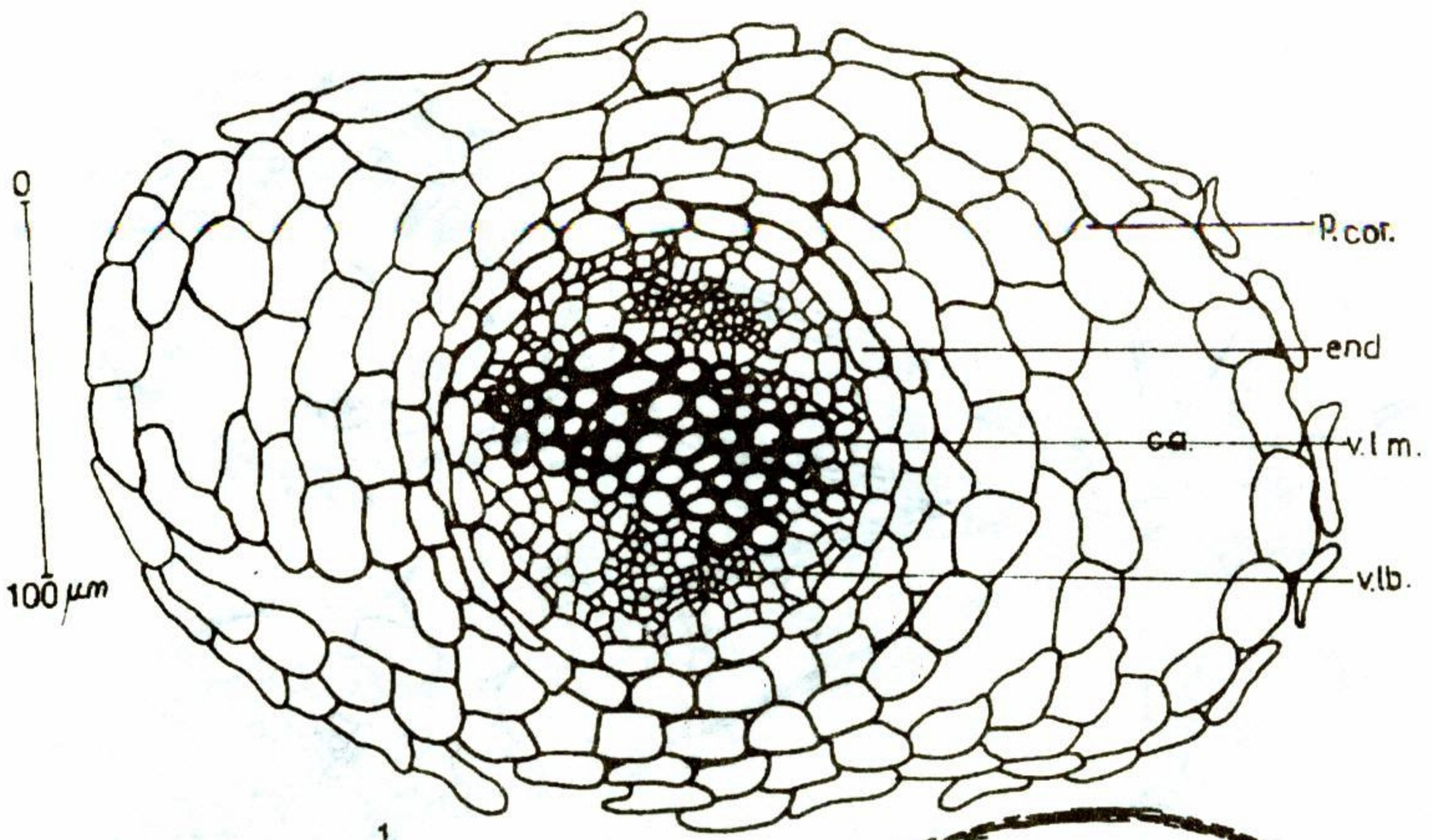
**The lamina.** Epidermis in surface view has isodiametric cells with straight lateral walls. Ranunculaceous type stomata are seen from place to place in both epidermis, so the lamina is amphistomatic. Star-shaped covering hairs are also in both epidermis. The lower epidermis has cells with lateral waved walls.

The midvein is prominent at the lower part with a single collaterally type bundle, in cross-section. The general structure is like that from the ocrea and petiole.

Prominent stomata are seen at the external level and numerous star-shaped covering hairs are in both epidermis.

The mesophyll is of palisadic type, under both epidermis, so the lamina has a bifacial-equifacial structure. The palisade-tissue has lower cells with lateral slightly waved walls, two-layered on the upper face and one-layered on the lower face.

**Plate I** – Cross-section of the root: Fig. 1 – detail; Cross-section of the rhizome: Fig. 2 – diagram, Fig. 3 – detail of vascular tissue, Fig. 4 – detail of cortical parenchyma, Fig. 5 – detail of medullar parenchyma.

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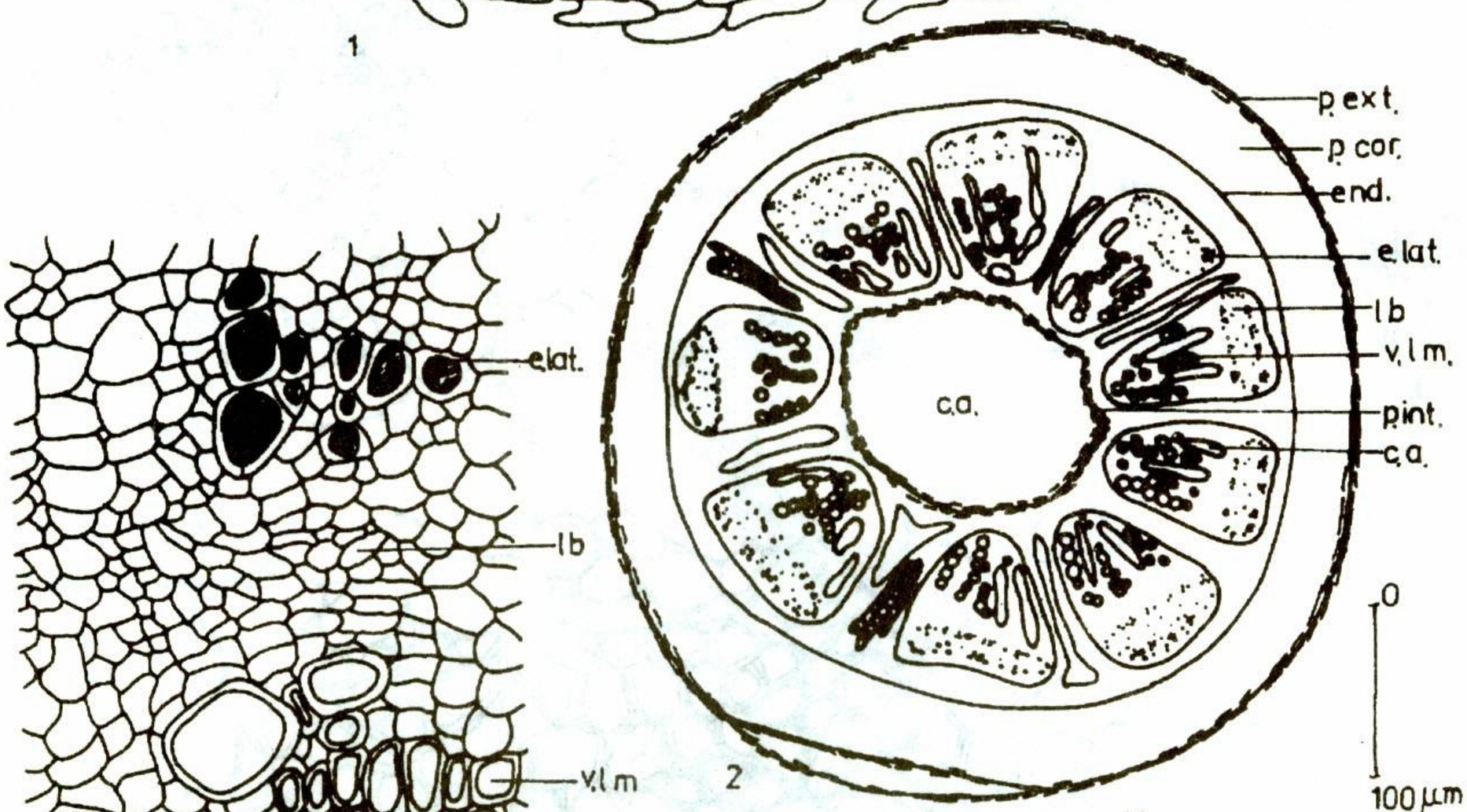


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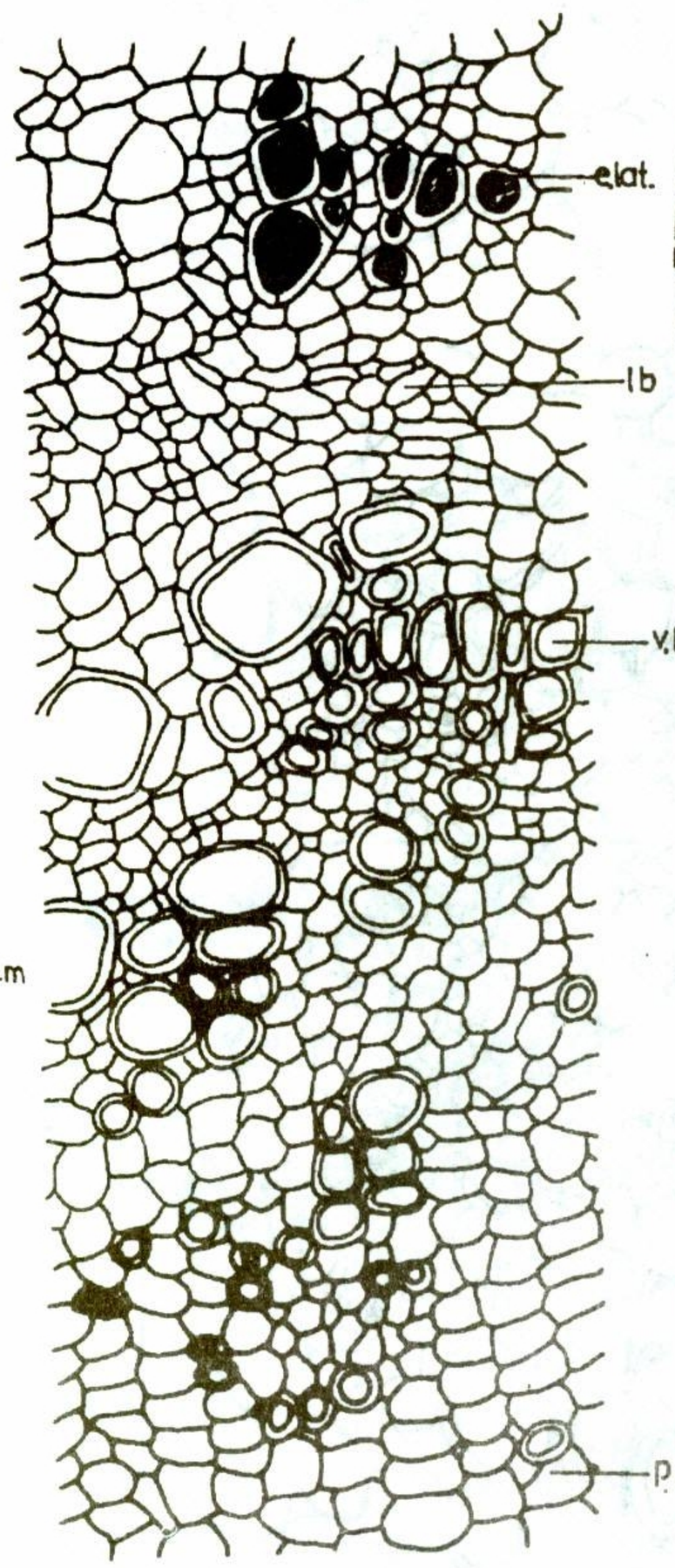


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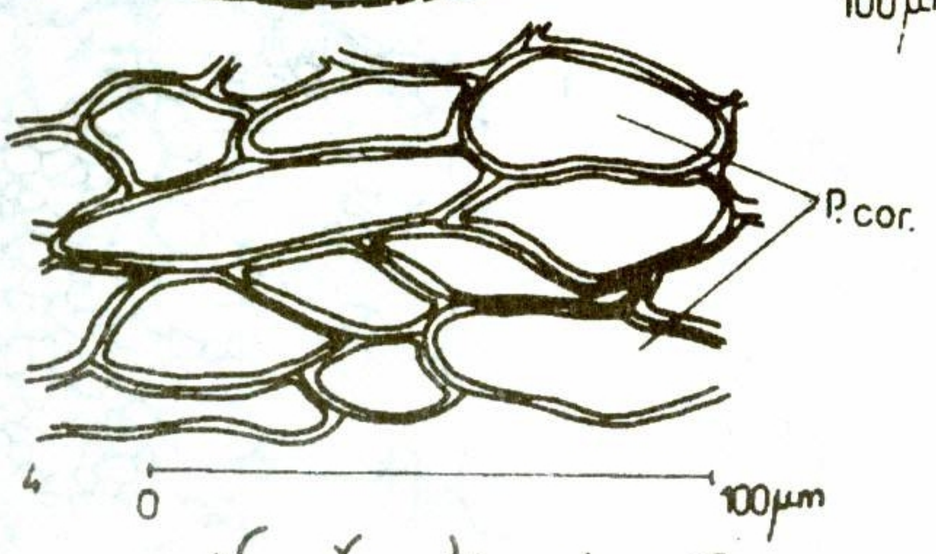


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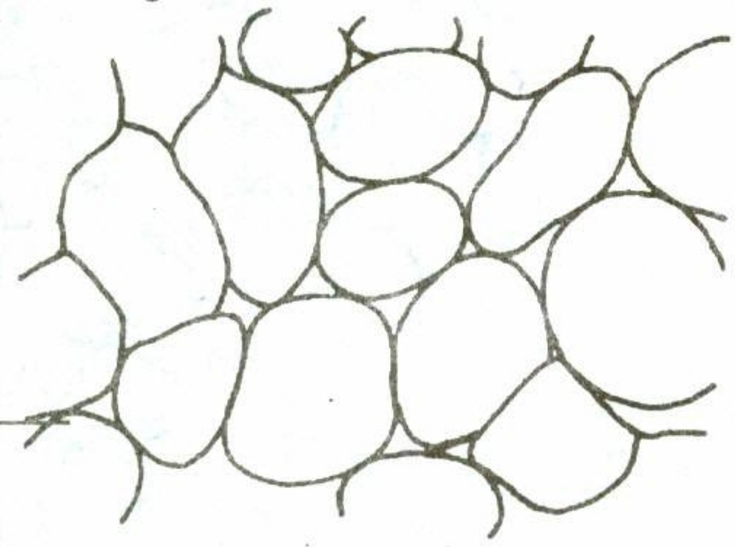


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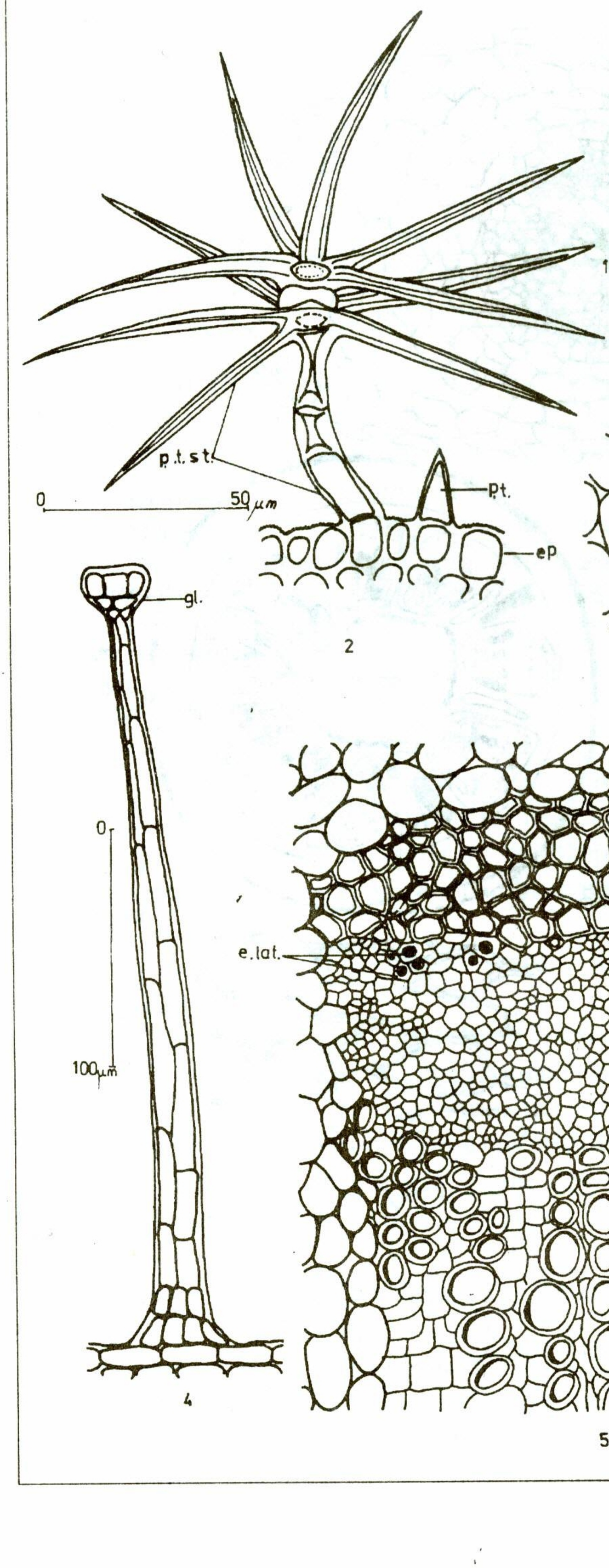
**Plate II** – Cross-section of the aerial stem: Fig. 1 – diagram, Fig. 2 – detail of epidermis with star-shaped covering hair and monocellular covering hair, Fig. 3 – detail of a cortical bundle, Fig. 4 – detail of a glandular hair, Fig. 5 – detail of vascular bundle from the central-cylinder.

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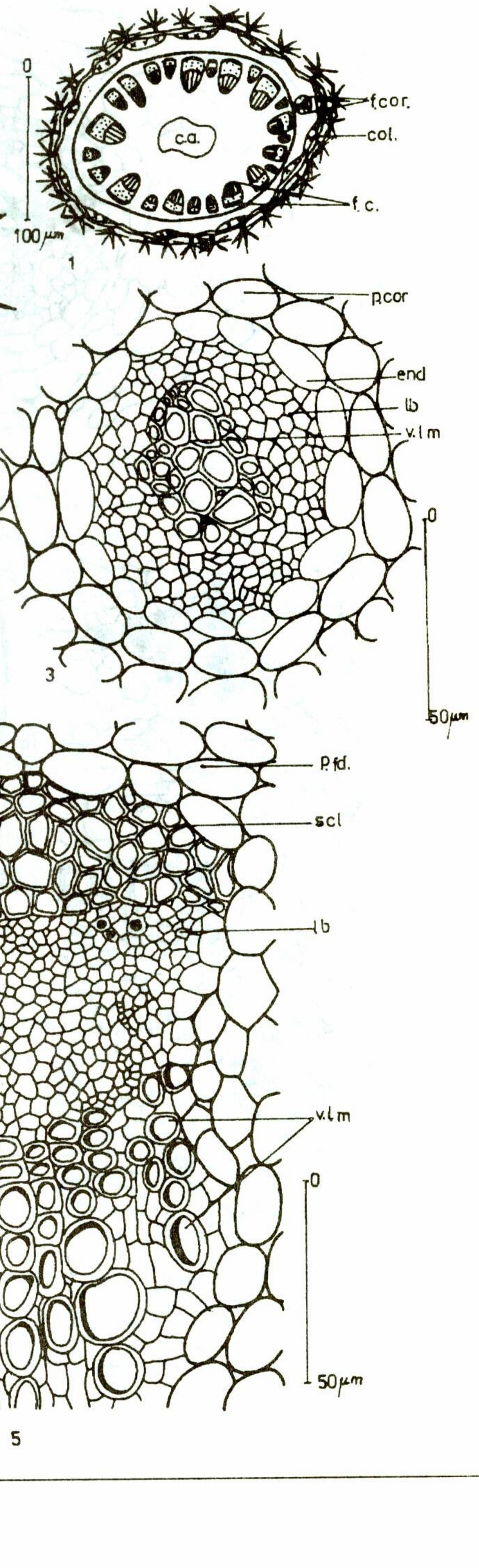


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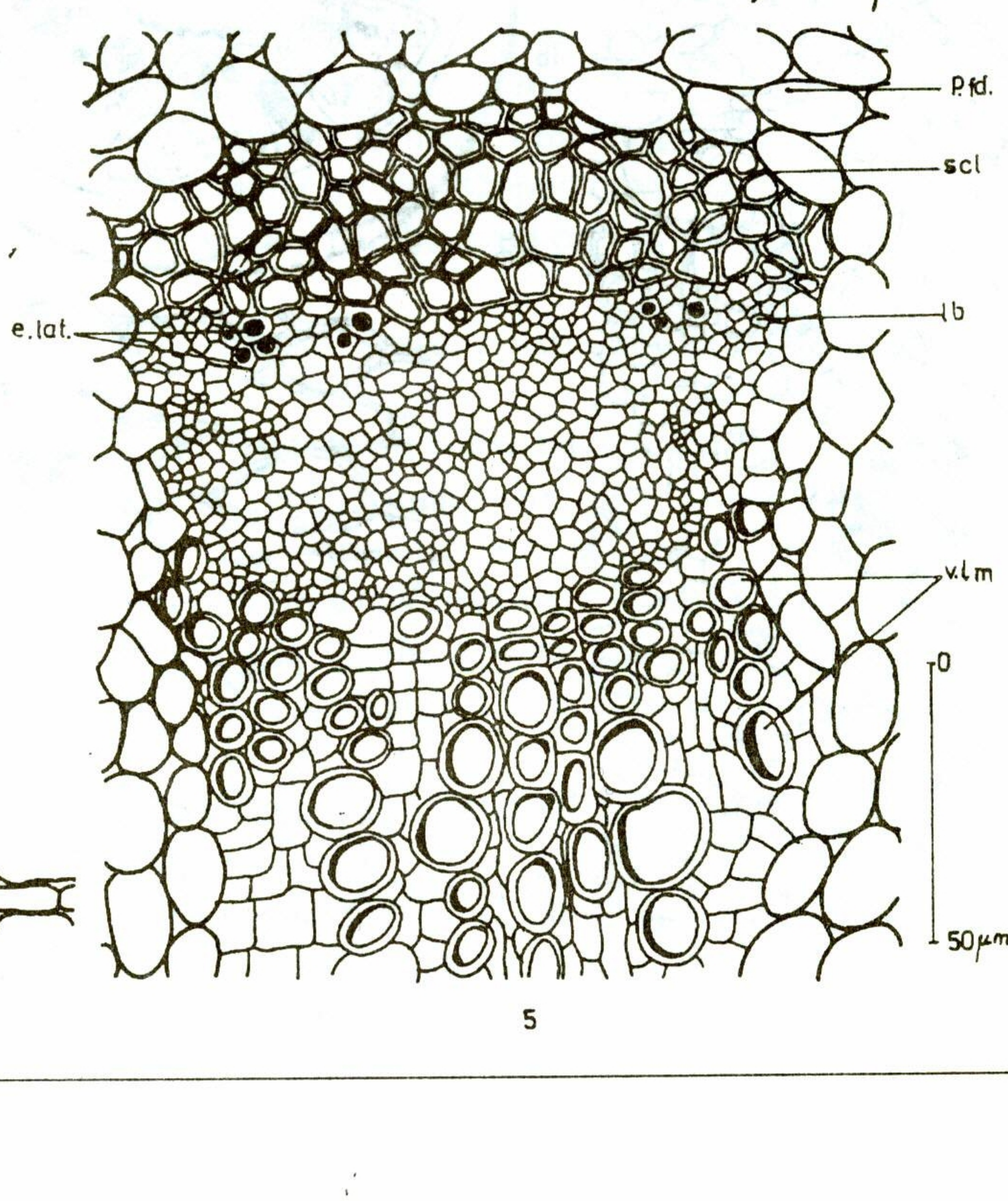


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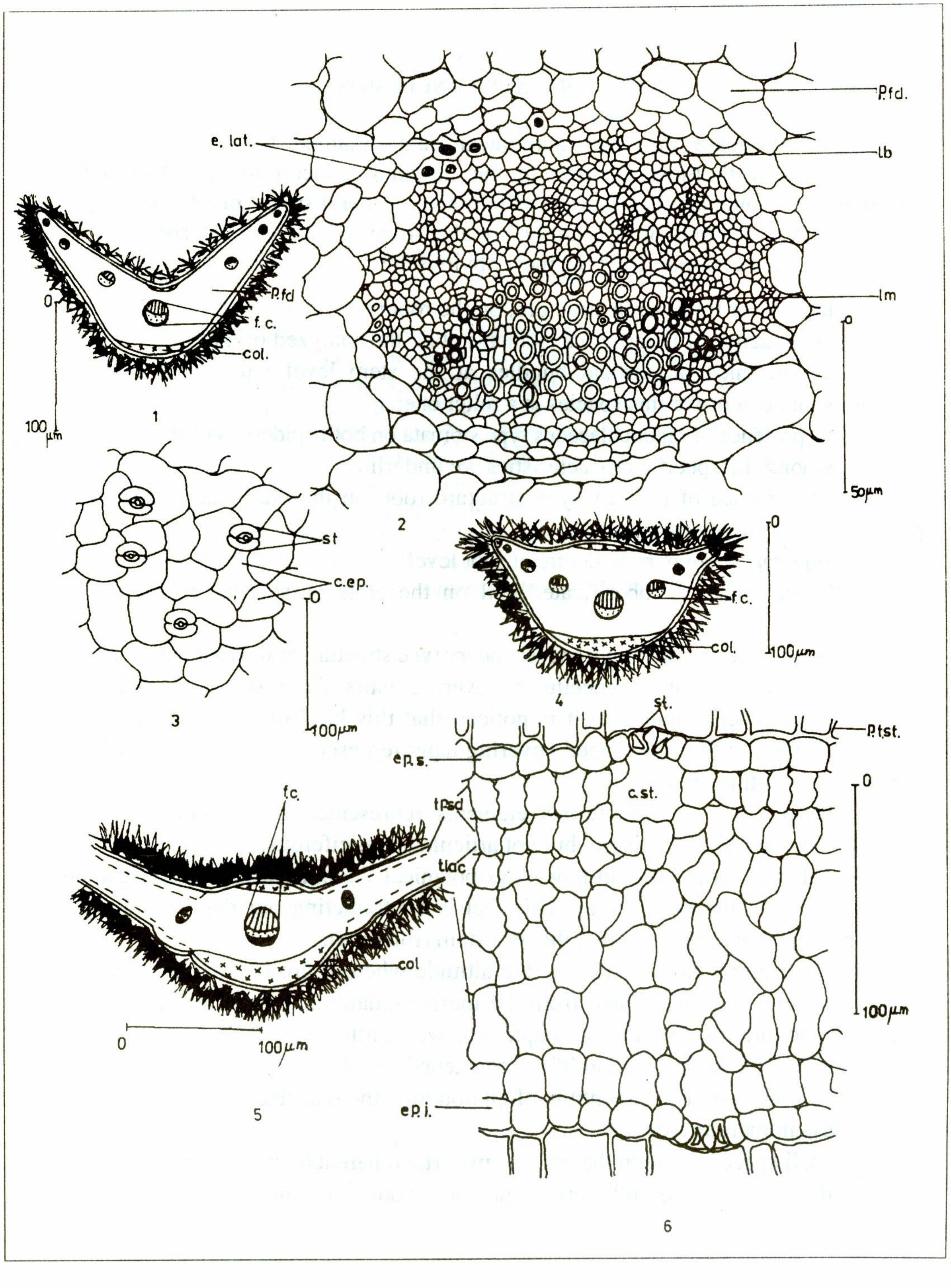
**Plate III** – Cross-section of the ocrea: fig. 1 – diagram, fig. 2 – detail of vascular bundle; Superficial section of the lamina: fig. 3 – detail of epidermis; Cross-section of the petiole: fig. 4 – diagram; Cross-section of the lamina: fig. 5 – diagram, fig. 6 – detail between the veins.



The spongy-tissue has isodiametric slightly tangential elongated cells with a small meatus between them.

## DISCUSSION AND CONCLUSIONS

If we compare the histo-anatomical data obtained by us at *Andryala levitomentosa,* with those existing in the literature concerning the *Asteraceae* family we can conclude that this species has some general structural characteristics common to the members of this family, but also some specific characteristics.

Among the common ones the most obvious are:

- the two- or three -arch structure, on the root level;
- the presence of laticiferous elements at all the analyzed organs;
- the presence of cortical bundles at the stem level which are vestigial structures that can prove this species is a relict one;
- the presence of ranunculaceous type stomata on both epidermis of the lamina.
- Among the specific characteristics we underline:
- the presence of primary type structure root, on the phloemic and cortical parenchyma, from the rhizome;
- abnormal secondary structure at root level;
- the presence of suberificated cell on the edge of xylemic tissue of the rhizome;
- the absence of libriform from secondary type structure of the root and stem;
- the presence of monocellular covering hairs from the stem and leaf epidermis (in specific literature it is noticed that this kind of hairs are absent at *Asteraceae* family; the star-shaped covering hairs represent a peculiar structure for the members of this family);
- the presence of laticiferous elements represented by secretory cells in association with phloemic tissue, but not articulated laticiferous.

We also underline that in nowadays botanical literature a thick and ligneous root is described for this species. This fact is contradicting our data because the structure of this organ proves that this is a stem (rhizome).

On the other hand, considering the altitude where it grows, we can conclude that this species is well adapted from the morpho-anatomical point of view at the ecological conditions. From these adaptations we mention:

- a fine-streaked with moniliform thickened cuticle;
- the presence of a tomentum, often doubling the real size of the lamina;
- less numerous stomata;
- small spaces between the cells from cortical parenchyma and mesophyll;
- the presence of assimilatory tissue under both epidermis.

**Abbreviations: c.a.** – air cavity; **c. ep.** – epidermal cell; **c. st.** – stomatic opisthial room; **col.** – collenchyma; **ep.** / epidermis (**i** – lower, **s** – upper); **e.lat.** - laticiferous elements; **end** – endodermis; **f.c.** – vascular bundle; **f. cor** – cortical bundle; **gl.** – gland; **lb** – phloem; **lm** – xylem; **p. cor** – cortical parenchyma; **p.ext.** – external periderm; **p. fd** – fundamental parenchyma; **p.int.** – internal periderm; **p.med.** – medullary parenchyma; **sb.** – suber; **scl.** – sclerenchyma; **st.** – stomata; **t. lac.** – spongy tissue; **t. psd.** – palisade parenchyma; **p.t.** – monocellular covering hair; **p.t.st.** – star-shaped covering hair; **v.lb.** – phloemic bundle; **v.lm.** – xylemic bundle.

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# DONNÉES CONCERNANT L'ANATOMIE DU *OREOPTERIS LIMBOSPERMA* (BELLARDI EX ALL.) J. HOLUB [*THELYPTERIS LIMBOSPERMA* (BELLARDI EX ALL.) H.P. FUCHS]

DANIELA DORIN, CONSTANTIN TOMA

Nous présentons dans ce travail quelques résultats concernant l'anatomie du *Oreopteris limbosperma*, plus exactement la structure des racines adventives, du rhizome, du pétiole, du rachis et de la lacinière, dans des stades ontogénétiques juvéniles et chez le sporophyte adulte, suivant en particulier la formation des éléments conducteurs et les traces des faisceaux libéro-ligneux au long des organes végétatifs.

## INTRODUCTION

Jusqu'à présent ont été publiés de nombreux ouvrages relativement à la structure des organes végétatifs et de reproduction des fougères, ainsi que résulte tout d'abord de l'ouvrage avec caractère de monographie publié par Ogura (1938). Nous même avons fait des études concernant l'anatomie des quelques espèces de fougères, en analysant la stomatogénèse et le complexe stomatal matur aux différents taxons (5), la structure des organes végétatifs du *Blechnum spicant* (7) et d'*Asplenium ceterach* (6). En ce qui concerne l'espèce *Oreopteris limbosperma* (Fam.*Thelypteridaceae*, Cls. *Filicopsida*), nous n'avons pas trouvé un ouvrage spécial au sujet de la structure de ces organes végétatifs. Des données sommaires et sporadiques on a trouvé dans quelques manuels et traités de botanique générale (1–3) ou d'anatomie végétale (4,8). Dans l'ouvrage publié de Pelourde (1906) on fait des références restreintes au nombre et à la forme de la masse ligneuse des faisceaux conducteurs du pétiole de la fronde de notre espèce. Évidemment, le plan général de structure des organes végétatifs du *Oreopteris limbosperma* est semblable avec celui des autres espèces de *Filicatae*, mais une description détaillée pour tous les organes, au cours de l'ontogenèse, n'a pas été réalisée jusqu'à nos jours.

## MATÉRIEL ET MÉTHODE DE TRAVAIL

Le matériel biologique analysé a été colecté de la Réserve Naturelle *Les Tourbières de Roûmont*, située entre Libin et Libramont, environ 6 km nord-ouest du Libramont, Province du Luxembourg, Belgique (approximativement 460 m altitude).

REV. ROUM. BIOL.–BIOL. VÉGÉT, TOME 45, N° 1, P. 71 – 82, BUCAREST, 2000

Pour obtenir les échantillons microscopiques on a utilisé la suivante méthode de travail: le matériel biologique (representé par des racines adventives, des rhizomes, des pétioles, des rachis et des lacinières, dans différents stades ontogénétiques) a été fixé chimiquement, puis déshydraté, imprégné en paraffine, sectionné au microtome Minot et les coupes (réalisées transversalement) ont été colorées avec methylene blue et ruthenium rot.

Toutes les observations et les dessins ont été faites au microscope MC1, après le miroir de projection.

## RÉSULTATS DE LA RECHERCHE

*La racine adventive* (Planche I)

Le rhizoderme a des cellules isodiamétriques, avec des parois minces; au stade adulte il commence à s'exfolier.

L'écorce est relativement mince (9–10 couches de cellules), ayant la couche externe formée par des cellules polygonal-isodiamétriques, avec les parois uniformément et légèrement épaissies, à commencer même des stades juvéniles, et impregnées avec une substance de type spécial (d'une nature chimique inconnue pour nous pour l'instant) qui leur donne des nuances de marron au brun-noirâtre. Les premières 2 couches du parenchyme fondamental ont des cellules avec des parois très légèrement épaissies et impregnées avec la même substance, principalement aux coins de la cellule. Le reste du parenchyme fondamental est typique, avec des cellules isodiamétriques, polygonales au début, mais arrondies ou légèrement allongées au stade adulte. La couche la plus interne de l'écorce est un endoderme de type initiale, les parois radiaires des cellules ayant les bandes de Caspary.

Le stèle est délimité par un péricycle bistratifié, avec des cellules grandes, isodiamétriques ou légèrement allongées tangentiellement par raport au stèle, avec toutes les parois minces.

Dans le stèle on peut observer deux petits vaisseaux du protoxylème, localisés diamétralement-opposés, entre lesquels on trouve une rangée diamétrale de quelques (5–7) grandes cellules, ayant initialement des parois minces, qui représentent de futures vaisseaux du metaxylème. Le grossissement et la lignification des parois commencent avec les petits vaisseaux du protoxylème, localisés près du péricycle et avancent vers le centre, ainsi qu'au stade adulte on peut distinguer deux faisceaux ligneux unis par les derniers vaisseaux formés, vaisseaux du métaxylème. Sur les deux parties du xylème il y a le phloème, avec des cellules criblées – initialement disposées immédiatement sous le péricycle, et cellules de parenchyme phloémique, plus grandes et avec les parois un peu plus minces.

*Le rhizome* (Planche II, III)

Le contour du rhizome, vu dans une coupe transversale, est irrégulier, déformé par les racines adventives et les pétioles qui en sortent; en faisant comparaison avec des autres espèces (par exemple *Thelypteris palustris*), le diamètre du rhizome est très petit.

L'épiderme est formé par de grandes cellules, isodiamétriques, avec des parois minces, celle externe étant légèrement bombée.

Les 4–5 couches de l'écorce ont des cellules isodiamétriques, avec les parois légèrement et uniformément épaissies et imprégnées avec la substance marron, comme les cellules du parenchyme fondamental, à l'exception des 2–3 couches qui entourent les faisceaux conducteurs.

Les faisceaux conducteurs, arrangés presque en cercle, au nombre très réduit (2–3) et de forme variable (circulaire ou allongée), sont du type hadrocentrique, chaqun étant délimité d'un endoderme de type initial et d'un péricycle uni- ou bistratifié, ayant ainsi la valeur d'un stèle.

La forme variable des faisceaux conducteurs du rhizome est due au détachement des faisceaux qui se dirigent vers la base des pétioles foliaires ou vers les racines adventives, en général la masse ligneuse ayant la forme générale du faisceau. Le xylème est consitué par des vaisseaux de calibre différent, séparés par de rares cellules de parenchyme ligneux, tandis que le phloème présente des cellules criblées et cellules de parenchyme phloémique.

## LA FRONDE

*Le pétiole* (Planche IV, V)

Sur des coupes transversales, le contour du pétiole est variable en fonction du niveau du sectionnement: demi-circulaire avec la face supérieure plane – à la sortie du rhizome et plus haut, et demi-circulaire avec deux côtés adaxiales et un large fossé entre eux – à la partie distale du pétiole.

Au bord, dans des stades ontogénétiques juvéniles, au niveau distal du pétiole, on peut observer des poils unicellulaires piriformes.

L'épiderme a des cellules isodiamétriques, avec des parois minces, celle externe étant légèrement bombée.

L'hypoderme est représenté par une ceinture pluristratifiée (1–4 couches), plus épaisse à la face inférieure et aux deux côtés adaxiales, avec des cellules polygonales, isodiamétriques, avec des tailles différentes; leurs parois, minces dans le stade juvénile, s'épaisissent uniformément dans le stade adulte, en formant un sclérenchyme typique. Le grossissement et la lignification des parois commencent à partir des couches hypodermiques plus internes et avancent vers l'épiderme.

Au niveau basal, dans le parenchyme fondamental on peut observer deux faisceaux conducteurs, petits et arrondis à la sortie du rhizome, grands et allongés plus haut, typiquement hadrocentriques, avec le xylème hypochamp, chaqun d'entre eux étant entouré d'un endoderme de type initial et d'un péricycle uni- ou bistratifié. Au long du pétiole, ces deux faisceaux se joignent en formant un seul faisceau conducteur qui a la forme de la lettre V, ses bras ayant la partie moyenne et les extrémités courbées vers l'intérieur. Le xylème est constitué des vaisseaux de calibre différent et des rares cellules de parenchyme ligneux, toutes étant entourées par l'anneau du phloème, avec des cellules criblées et cellules de parenchyme phloémique. Le nombre des pôles ligneux est variable:

- chacun des deux faisceaux de la base du pétiole a deux pôles ligneux: un pôle adaxial et un pôle abaxial;
- dans le stade où les faisceaux commencent à se joindre, on a 4 pôles ligneux;
- à partir du moment quand on a un seul faisceau (à la partie distale du pétiole et au long du rachis) on trouve 3 pôles ligneux: un pôle abaxial et deux pôles adaxiaux.

Les premières cellules criblées apparaissent initialement sur les deux parties du xylème, au début vers l'extérieur du xylème, puis aussi vers l'intérieur, et à l'intérieur initialement vers la partie supérieure, puis aussi vers celle inférieure.

*Le rachis* (Planche IV)

En général, au stade juvénile ainsi qu'au stade adulte, le rachis garde le contour et la structure de la partie distale du pétiole: épiderme, hypoderme sclérenchymatique à la face abaxiale et aux deux côtes adaxiales, parenchyme fondamental et un seul faisceau conducteur avec la forme de la lettre V; au long du rachis, les extrémités de ce faisceau séparent de nouveaux petits faisceaux qui se dirigent vers les lacinières.

*La lacinière* (Planche VI)

Les cellules épidermiques sont isodiamétriques ou allongées tangentiellement, de taille différente (toujours plus grandes à la face supérieure), celles de l'épiderme supérieure ayant la paroi externe un peu plus épaissie; vus sur une coupe transversale, les stomates sont proéminents au-dessus du niveau externe de l'épiderme.

À la face inférieure de la lacinière, aux stades juvéniles, on peut observer des poils bicellulaires avec la cellule terminale très grande, arrondie.

Le mésophylle, aux stades juvéniles, est formé par des cellules polygonales en voie de différenciation, qui ne laissent pas des espaces aérifères entre eux; dans ce mésophylle on trouve des faisceaux du procambium, formés par des cellules polygonales petites, avec un grand noyau central. Plus tard, à partir de la moitié

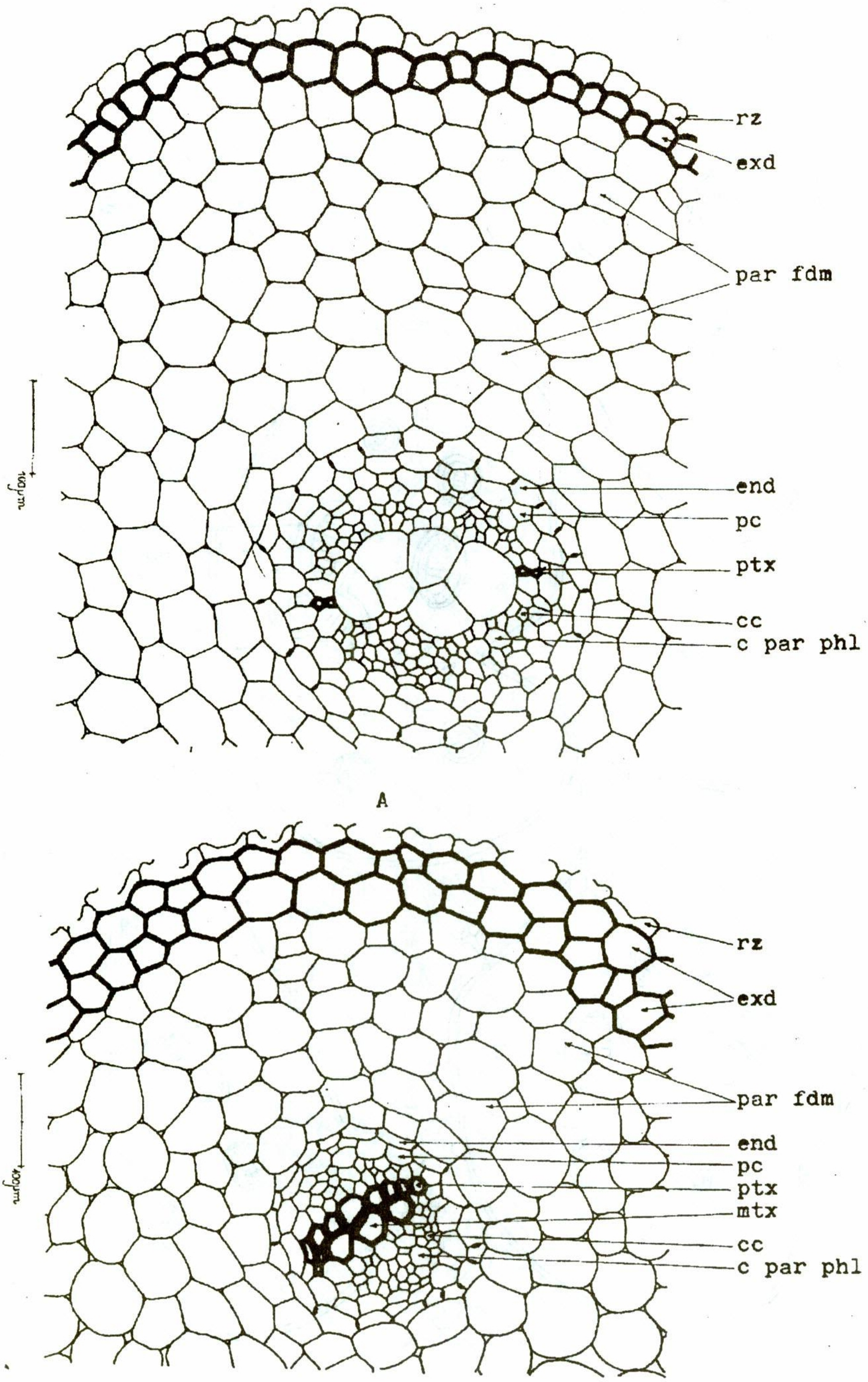


**Planche I** – Détails des coupes transversales dans la racine adventive au stade juvénile (A) et adulte (B).

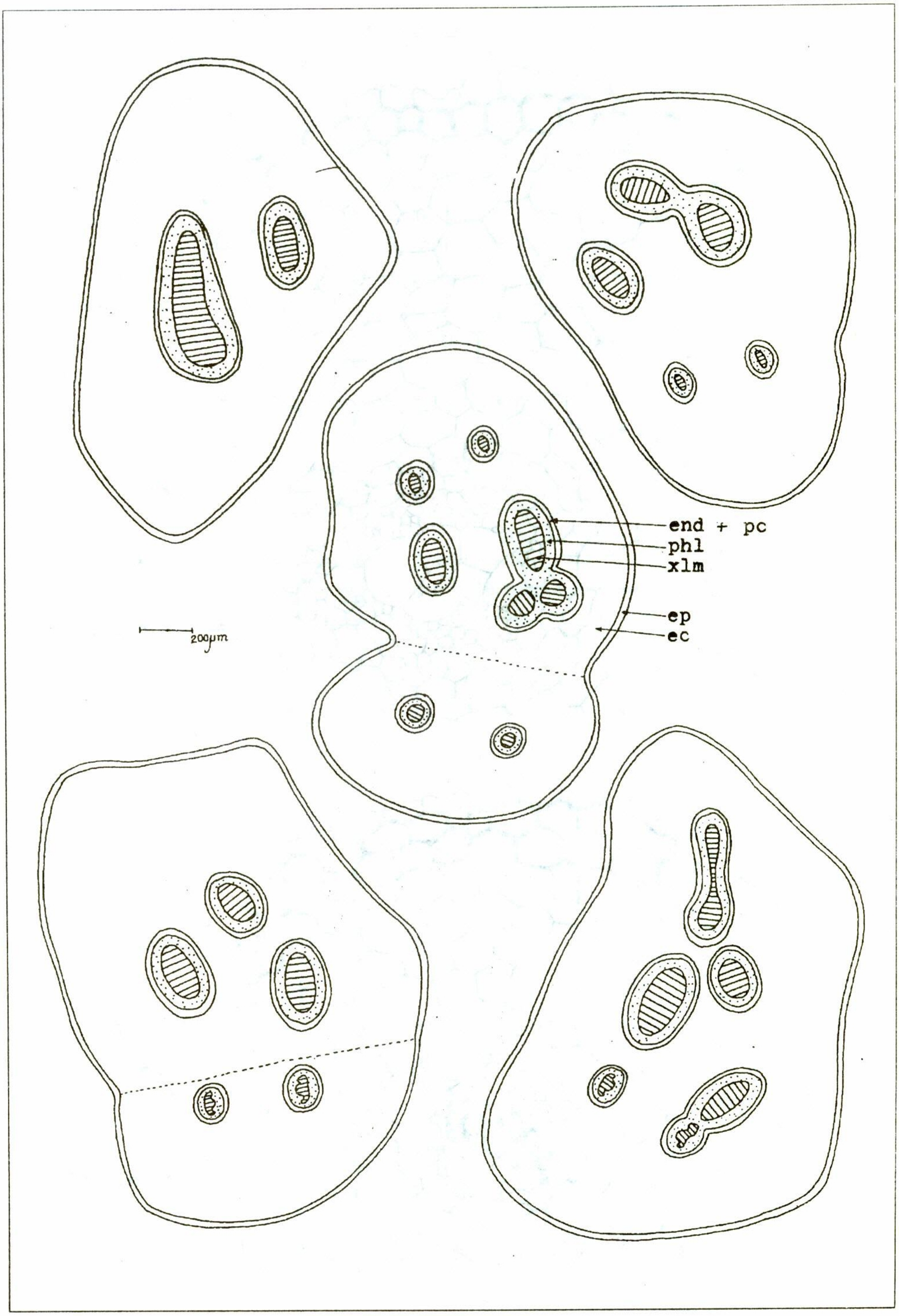


**Planche II** – Schémas des coupes transversales dans le rhizome.

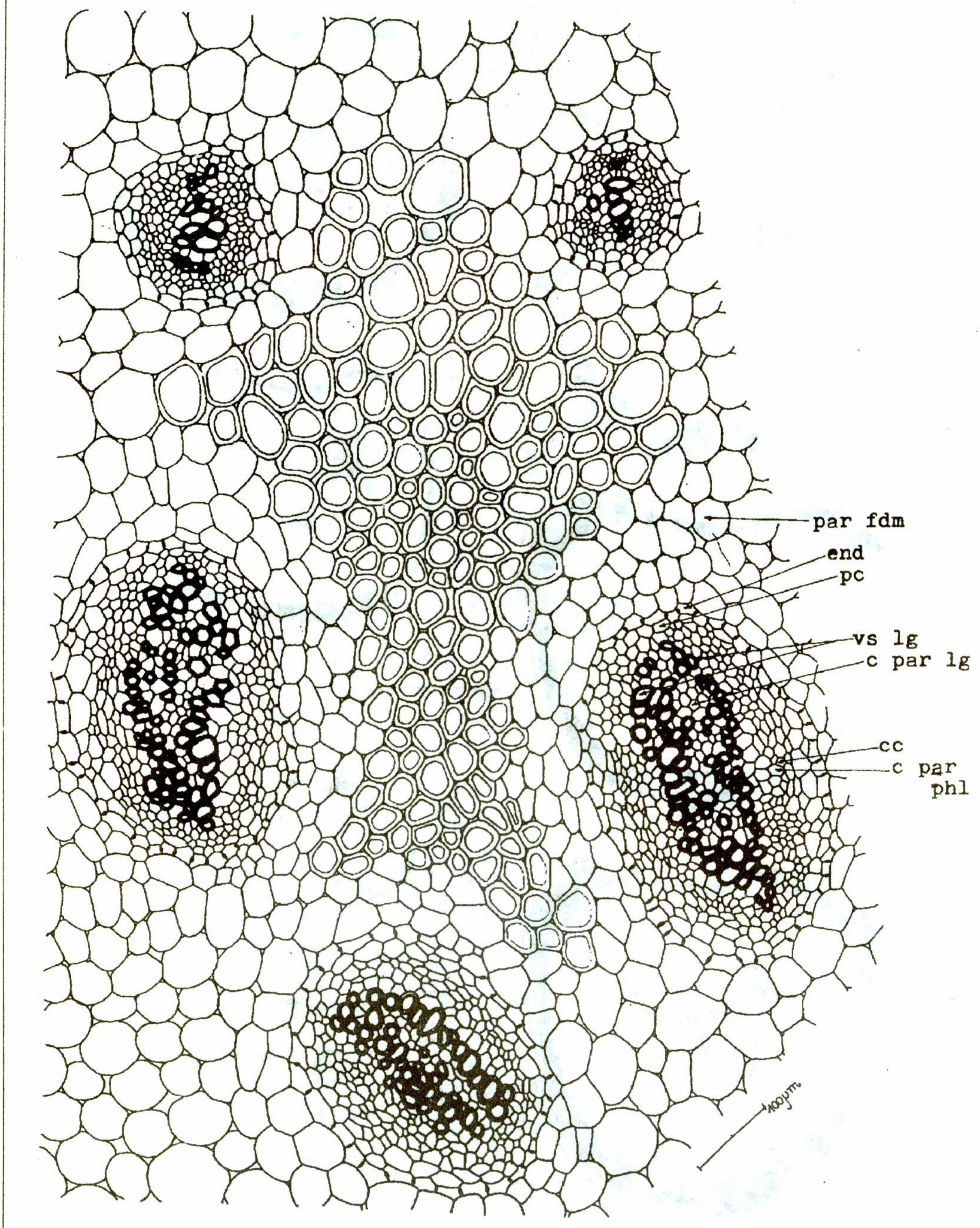


**Planche III** – Détail de la coupe transversale dans le rhizome.

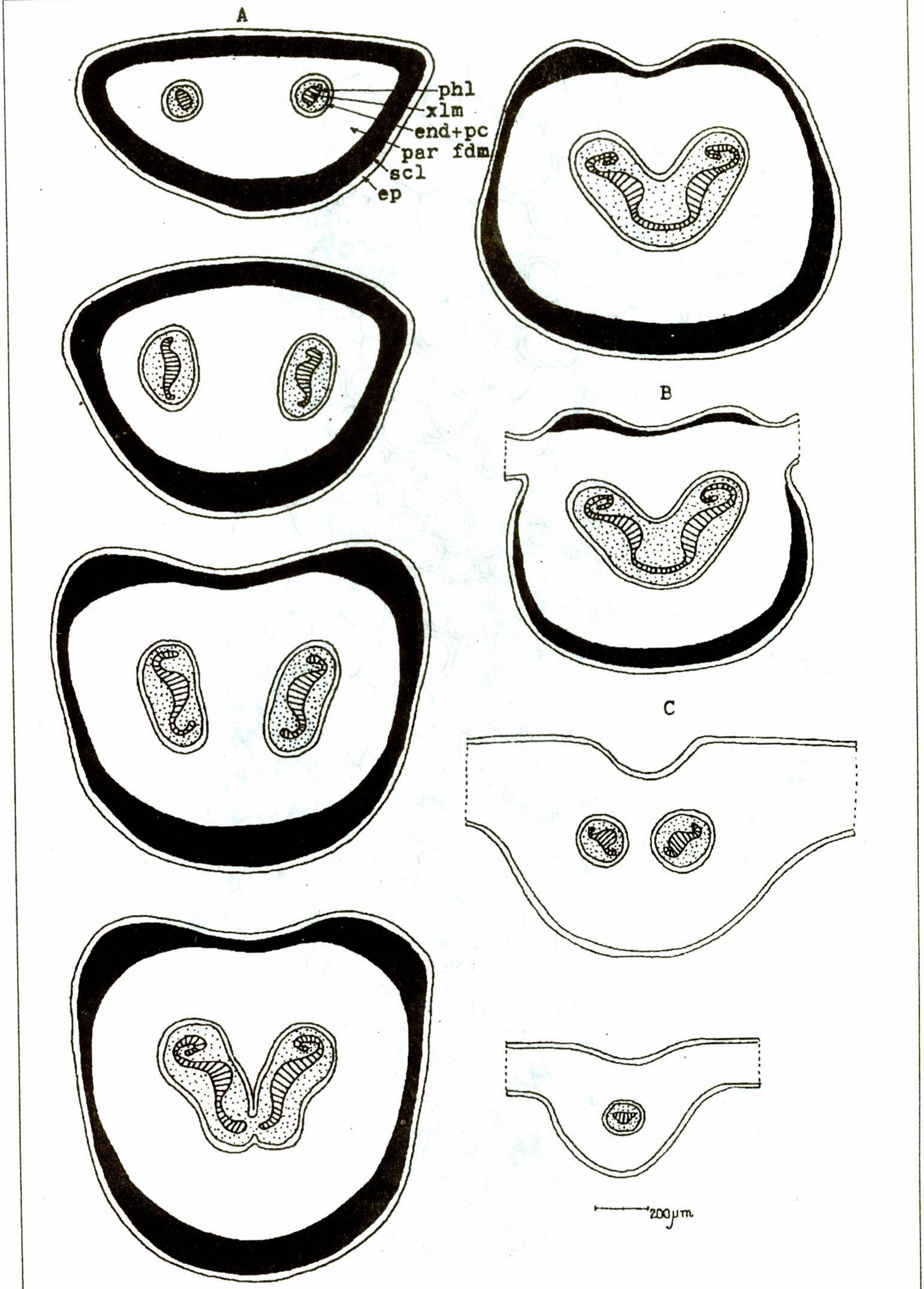


**Planche IV** – Schémas des coupes transversales au long du pétiole (A), dans le rachis (B) et dans la nervure médiane de la lacinière (C).

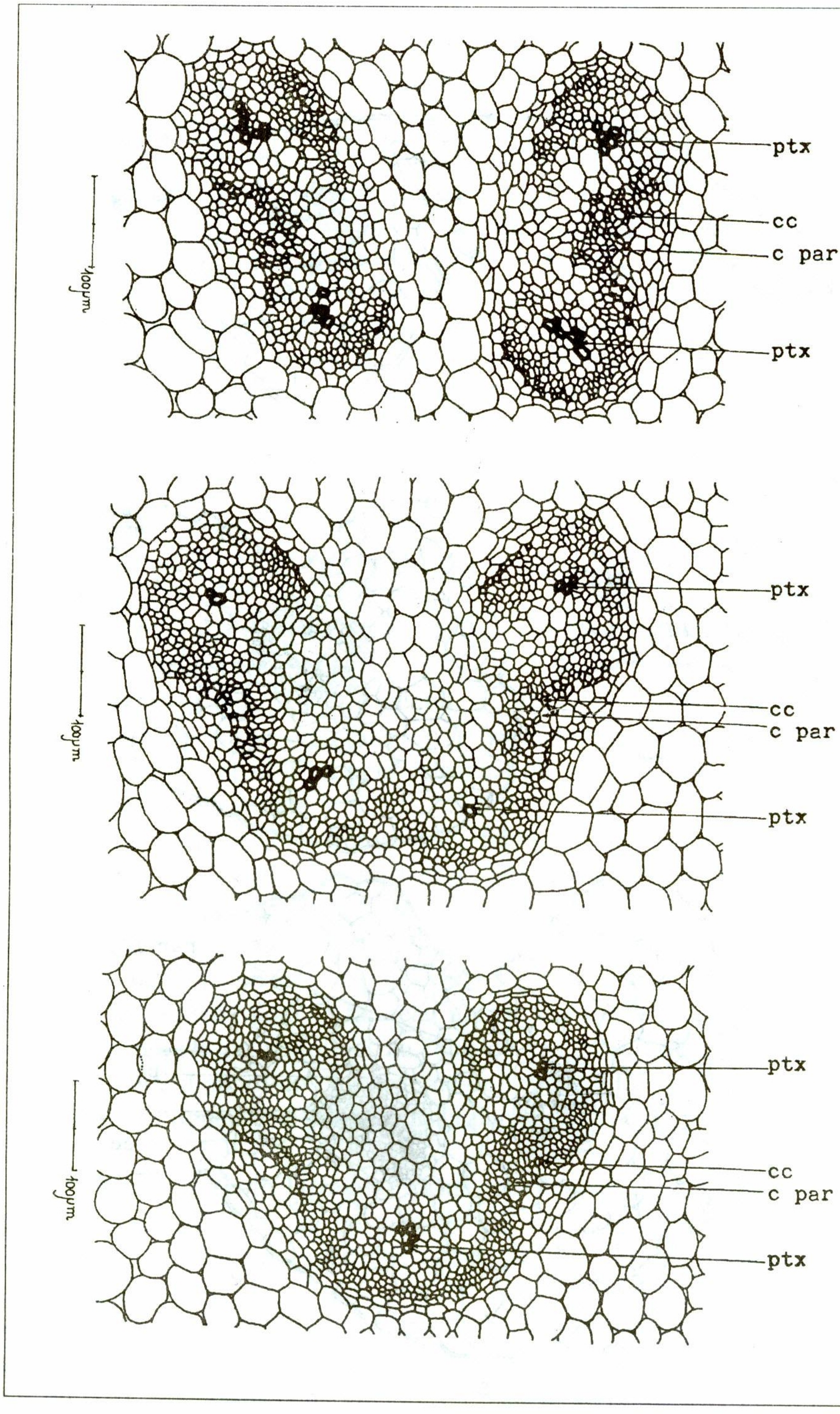


**Planche V** – Détails des coupes transversales au long du pétiole, au stade ontogénétique juvénile.

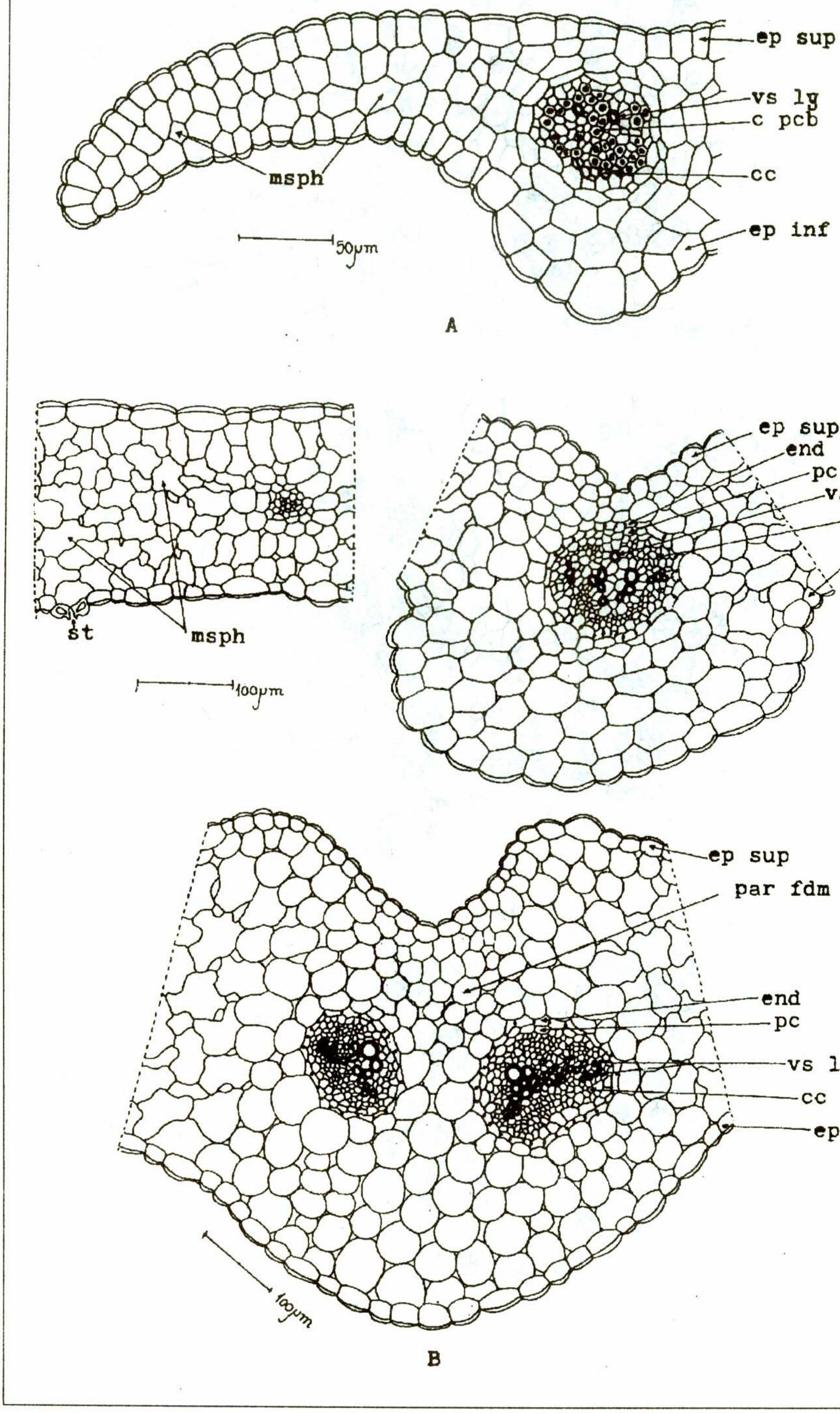


**Planche VI** – Détails des coupes transversales dans la lacinière très jeune (A) et définitive (B, C).

inférieure du mésophylle, on peut observer l'apparition de petits méats, qui augmentent graduellement, pendant que les cellules prennent une forme lobée. Le mésophylle de la lacinière adulte est homogène, de type lacuneux, avec des cellules lobées, plus compactes vers la face supérieure.

Observée en coupe transversale, la nervure médiane de la lacinière est proéminente à la face inférieure, avec un large fossé adaxial, et présente un ou deux faisceaux conducteurs. Les premiers vaisseaux ligneux se forment vers la partie supérieure de la lacinière et les premiers éléments phloémiques – vers celle inférieure; donc, on peut dire que, initialement, les faisceaux conducteurs foliaires sont de type collatéral, mais ultérieurement le phloème entoure la masse ligneuse pour former le faisceau hadrocentrique caractéristique pour la structure définitive de la lacinière, entouré d'un péricycle uni- ou bistratifié et d'un endoderme de type initial.

## CONCLUSIONS

- Le stèle des racines adventives est de type diarche, le xylème et le phloème ayant un développement centripète.
- Le rhizome a un diamètre réduit et un petit nombre de faisceaux conducteurs (2–3) de forme variable, du type hadrocentrique, délimités d'un endoderme de type initial et d'un péricycle uni- ou bistratifié, ainsi que chacun d'entre eux a la valeur d'un stèle.
- À la base du pétiole on trouve deux faisceaux conducteurs typiquement hadrocentriques, avec le xylème hypochamp, qui se joignent au long du pétiole pour former un seul faisceau ayant la forme de la lettre V; le nombre des pôles ligneux est variable, suivant la situation: deux pôles pour chacun des deux faisceaux de la base du pétiole, 4 pôles quand les faisceaux commencent à se joindre et 3 pôles ligneux pour le faisceau unique, en forme de V, qui se trouve vers la partie terminale du pétiole et au long du rachis. L'ordre d'apparition de cellules criblées est: d'abord du côté externe de la masse ligneuse et puis du côté interne de la masse ligneuse: d'abord vers la partie supérieure, puis vers la partie inférieure.
- Le mésophylle de la lacinière, formé initialement par de petites cellules polygonales, méristématiques ou en voie de différenciation, au stade adulte est homogène, de type lacuneux, avec des cellules lobées, plus compactes vers la partie supérieure.
- Les faisceaux conducteurs foliaires sont initialement de type collatéral (les premiers vaisseaux ligneux se forment vers la partie supérieure et les premiers éléments phloémiques – vers celle inférieure), mais ils deviennent hadrocentriques dans des stades du développement plus avancés.

**Abreviations dans les planches I-VI:**

c.c. – cellules criblées
c.par. – cellules parenchymatiques
c.par.phl. – cellules de parenchyme phloémique
c.par.lg. – cellules de parenchyme ligneux
c.pcb. – cellules de procambium
ec. – écorce
end. – endoderme
exd. – exoderme
ep. – épiderme
ep.inf. – épiderme inférieur
ep.sup. – épiderme supérieur
msph. - mésophylle
mtx. - métaxylème
par.fdm. – parenchyme fondamental
pc. – péricycle
phl. – phloème
ptx. – protoxylème
rz. – rhizoderme
scl. – sclérenchyme
st. - stomata
vs.lg. – vaisseaux ligneux
xlm. – xylème

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# THE INFLUENCE OF THE METRIBUZIN HERBICIDE ON GROWTH, GASEOUS EXCHANGES AND ASSIMILATORY PIGMENT CONTENT IN *BOTRYOCOCCUS BRAUNII*

DANIELA ANCA LAZĂR

The reactions of *Botryococcus braunii* Kuetz. cells treated with a triazine derivative, metribuzin, were investigated. The effects on cell numbers, growth, assimilatory pigments content and gas exchanges were recorded.

Metribuzin herbicide is a triazine derivative and it was added in Zehnder-Gorham nutritive medium of algal suspension in increasing concentrations from 0.02 to 0.6 μM.

Compared with the control, the 0.2 μM metribuzin concentration reduced the growth, photosynthesis and respiration down to 51.15, 51.7, and respectively 56.3% (average percentages). For the 0.2 μM treatment, the assimilatory pigments content showed a decrease below 50% of control value after 7 days for metribuzin.

The results show that the metribuzin herbicide induced a quick and sharp limitation of the physiological activity related with algal cell growth in liquid suspension.

*Key words*: *Botryococcus braunii*, metribuzin herbicide, growth, photosynthesis, respiration, assimilatory pigments.

## INTRODUCTION

Herbicides are used extensively today to eliminate unwanted competing species of plants in the cultivated environment (8).

This phytotoxic compounds block metabolic pathways essential for plant growth (9).

The herbicidal action of chemical compounds depends on their interaction with a multitude of major biochemical reactions in the plant. The majority of commercial herbicides attack the photosynthetic apparatus (2). Others interfere with respiratory processes, pigments synthesis (3) or growth reactions. Usually a herbicide affects more than one metabolic reaction in plants (5,6).

Photosynthesis, as the central series of metabolic reactions in plants, offers a preferential target site for a large number of herbicides. It is estimated that more than half of all commercial herbicides act primarily by interfering with photosynthetic reactions. This physiological process or plant pigment synthesis have no counterpart in animal physiology (10).

Phytotoxic symptoms occur during growth, and these effects can be advantageously followed and quantitated with (liquid) algae or cell cultures. Thus, the unicellular algae represent a useful experimental material for the study of the herbicide impact on plant physiology (1). Because of the great number of cells from the volume unit of the algal suspension, the effects of the individual variations are eliminated. Therefore, the unicellular algae represent a good biological material for the study of the herbicide influence on some physiological processes of both algae and higher plants.

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The reactions of *Botryococcus braunii* Kuetz.* cells treated with a triazine derivative, metribuzin (4-amino-6-1,1-dimethylethyl-3-(methylthio)-1,2,4-triazin-5-(4H)-one) (11) were investigated.

Metribuzin is a selective triazine herbicide which inhibits photosynthesis of susceptible plant species. It is used for control of annual grasses and numerous broadleaf weeds in field and vegetable crops, in turfgrass, and on fallow lands. Metribuzin as a pre-treatment can be applied before or after planting but must be applied before crop germinates (11).

The molecular formula for metribuzin is $C_8H_{14}N_4OS$ and its solubility in water at 20°C is 1.05 g/l (11).

For one culture cycle, the effects on cell numbers, growth, gas exchanges and assimilatory pigments content were recorded.

## MATERIALS AND METHODS

*Botryococcus braunii* alga was cultivated on Zehnder-Gorham nutritive medium (12). The experiments were carried out in a chamber with artificial illumination of 8000 lux. To avoid mutual shadowing, the algal suspensions, in cylindrical glass recipients of 1000 ml, were bubbled with steady stream of air produced by vibrator devices. The culture medium was inoculated with an amount of algal biomass producing a 100000 cells/ml suspension in all experiment variants. The ambient temperature varied between 24–25°C. The results represent average values of three repetitions for each variant.

The metribuzin herbicide was introduced in the culture medium before inoculation and the concentrations of the tested herbicide solutions varied from 0.02 μM to 0.6 μM.

The commercial product Sencor was used as a source of metribuzin. The Sencor herbicide has 70% active substance.

The growth of alga was observed at each two days within the culture cycle (established at 16 days) with a "Cecil 1020" spectrophotometer at $\lambda = 676$ nm. A linear regression was performed in order to produce a relationship between the extinction of the algal culture and the number of cells counted with Thoma haemocytometric mount for 24 samples from different treatments. The R squared was significant at a P value = 0.001 (Fig. 1). This relationship was used to convert the extinctions into a number of cells.

The areas ($A$) encompassed under the graphs for the number of cells/ml (X) from each concentration ($c$) at different days ($t$) were calculated with the following formula:

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* Isolated at the Biology Research Center of Cluj-Napoca from marshes of Sălicea (12).

$$A_{(c,t_n)} = \sum_{i=0}^{n} (t_{i+1} - t_i) \times \frac{(x_{(c,t_i)} + x_{(c,t_{i+1})})}{2}$$

for $t = 2$ and 14 days.

For each replicate, several simply non-linear regressions were performed between the reductions (% from control) of these areas ($X$ values) and the doses of herbicide as $Y$ values. The equation: $Y = a + bX^3$ was preferred because the confidence intervals were narrower than those obtained by other types of equations which produced better values for $R^2$. The equations for each replicate were used to interpolate the LD 0, 25, 50, 75, 100% for these replications in order to calculate the standard deviations. The regressions for all the replicates from a given day were used to calculate a common $R^2$. The interpolated values with these equations were similar to those obtained from replicates.

The photosynthesis rate was investigated with the Warburg method using the treated algal suspension and the Warburg buffer solution number 9 (15 ml $Na_2CO_3$ 0.1M + 85 ml $NaHCO_3$ 0.1 M)(4).

The respiration rate was established with the Warburg method using the treated algal suspension and a solution of KOH 30% to capture the $CO_2$ resulted in the respiration process (4).

The absorbance of the solution extracted with 100% acetone was measured at 661.6, 644.8 and 470 nm and the calculation of the content in a,b chlorophylls and carotenoids was performed according to Lichtenthaler (1987) (7); the values were expressed in mg/g dry matter (DM).

## RESULTS AND DISCUSSIONS

For the extinction curves of all treatments there is an obvious inflexion point, but the sizes of the slope decreasing after these points is in proportion with the applied dose of metribuzin. For the concentrations higher than 0.3 μM the growth depression is already installed after the second day from herbicide addition. The 0.02 μM treatment effect on the cell number is relatively close to control, but after the first 4 days these two curves are distinct. After the same period, the curve for 0.2 μM concentration is clear above the curve of 0.3 μM metribuzin (Fig. 2).

The averages for the metribuzin lethal doses for 2 and 14 days, calculated for each replicate as described in "Materials and methods", are presented in Table 1.

The metribuzin in 0.02 μM concentration had no inhibitory activity on photosynthesis intensity in the first period of the culture cycle, but soon, from the fourth day of the experiment an obvious depression appeared in the evolution of the photosynthesis (Fig. 3). The concentrations of metribuzin higher than 0.2 μM

obviously affected the photosynthesis in *Botryococcus braunii*. The concentration of 0.2 μM metribuzin reduced the photosynthesis to 51.7%. According to available literature, metribuzin moves to the site of photosynthesis. Metribuzin influences this physiological process at a concentration of 0.125–0.375 μM in *Bumilleriopsis filiformis* (1).

*Table 1*

Metribuzin lethal doses (μM) for *Botryococcus braunii*

| | 2 days | | 14 days | |
|---|---|---|---|---|
| **Death rate (%)** | **Average** | **SD*** | **Average** | **SD*** |
| 0 | 0.015 | 0.009 | 0.029 | 0.001 |
| 25 | 0.096 | 0.003 | 0.046 | 0.000 |
| **50** | **0.661** | 0.039 | **0.163** | 0.001 |
| 75 | 2.195 | 0.152 | 0.480 | 0.006 |
| 100 | 5.183 | 0.373 | 1.098 | 0.015 |
| | $R^2 = 0.975$ | | $R^2 = 0.982$ | |

*) SD = standard deviation

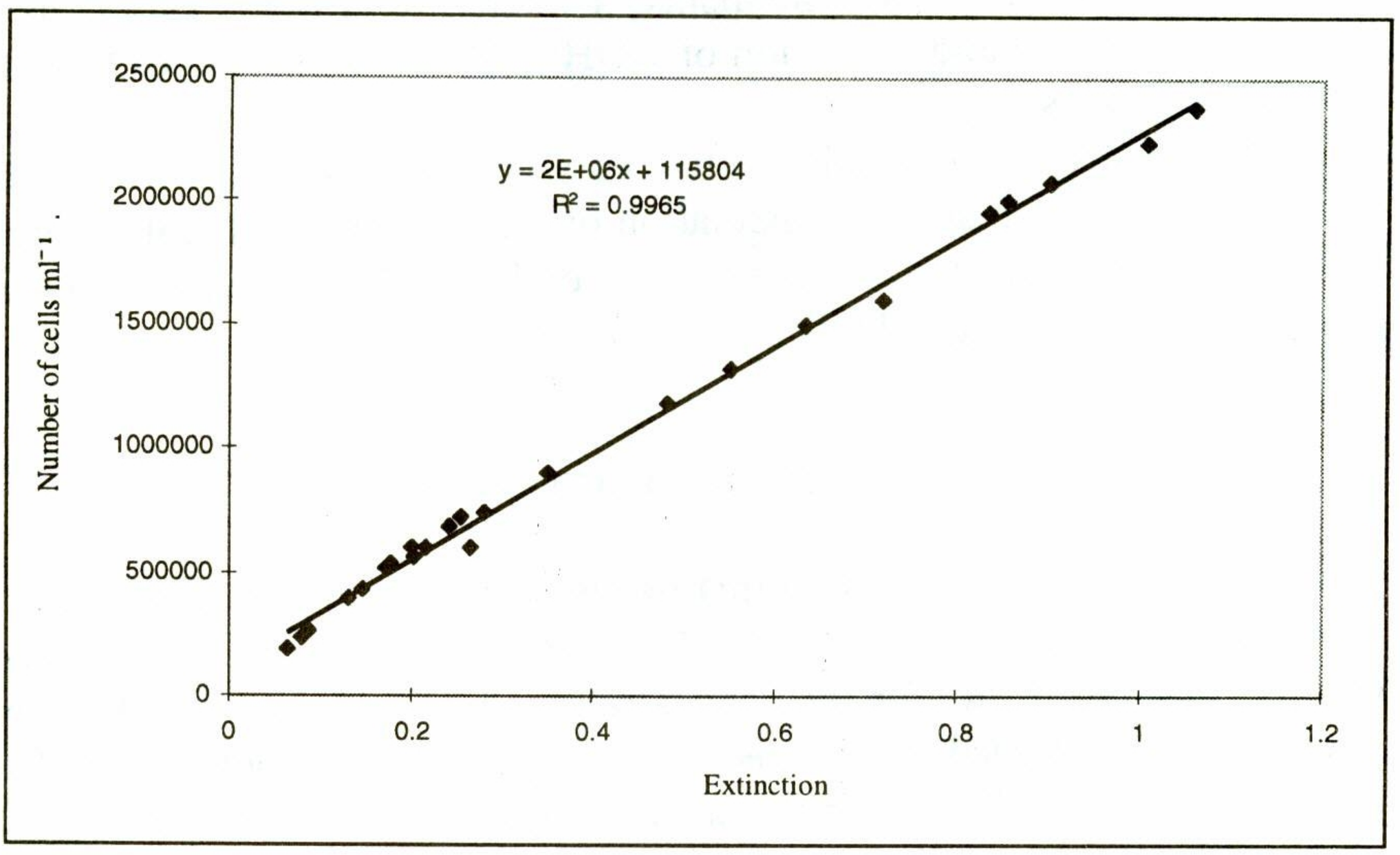


Fig. 1 – The relationship between extinction and the cells number/ml.

The metribuzin in concentrations of 0.2 μM to 0.6 μM produced a significant inhibitory effect on the oxygen absorption from the first day of the experiment. This inhibitory effect became larger in the last observation days. Compared with the control, the 0.2 μM metribuzin concentration reduced the respiration down to

56.3%. In the first period of the culture cycle, the values for the 0.02 μM treatment did not differ significantly from control (Fig. 4).

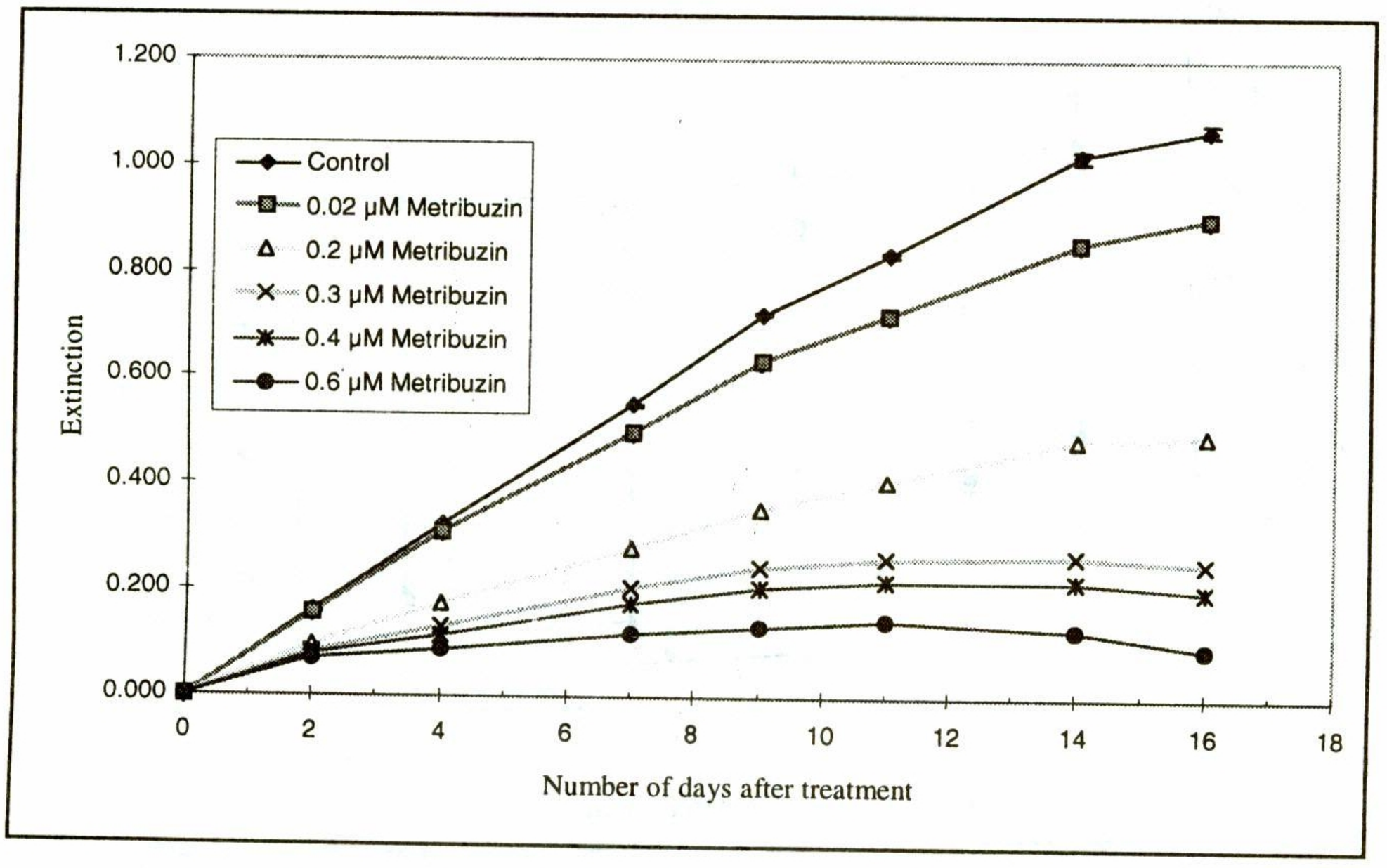


Fig. 2 – The influence of metribuzin on extinction of culture of *Botryococcus braunii.*

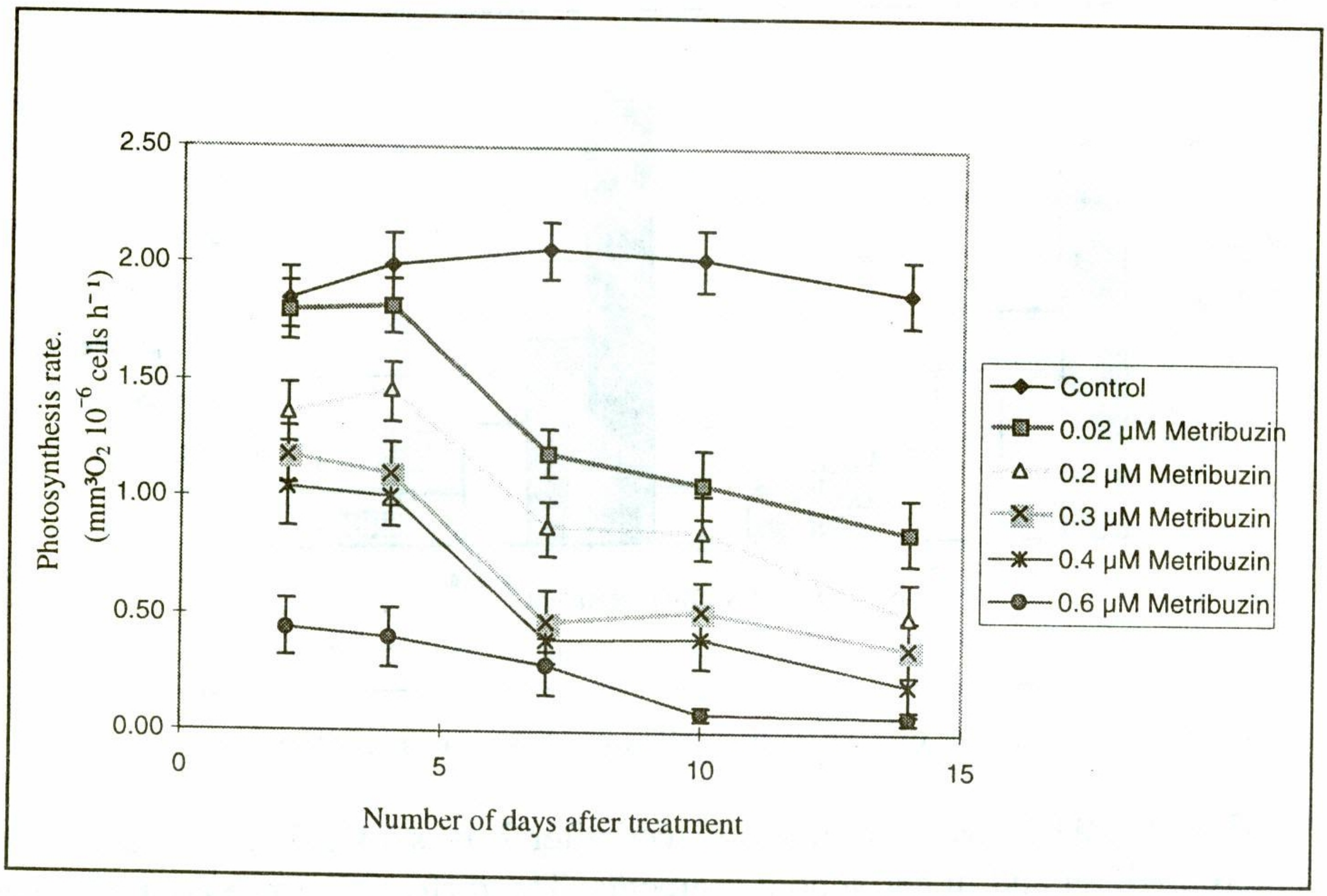


Fig. 3 – The influence of metribuzin on photosynthesis rate of *Botryococcus braunii.*

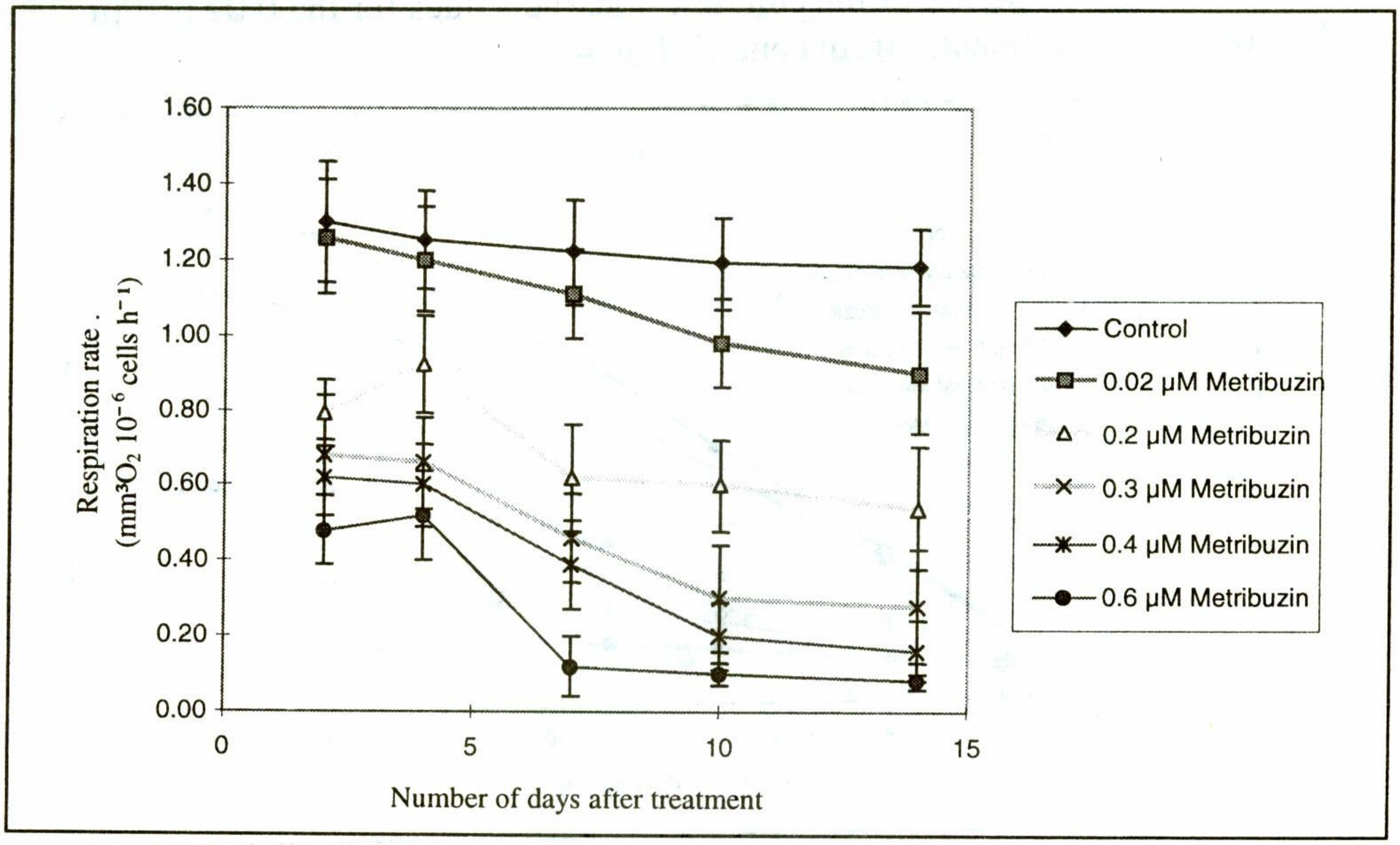


Fig. 4 – The influence of metribuzin on respiration rate of *Botryococcus braunii.*
(The vertical bars are equal to two standard deviations)

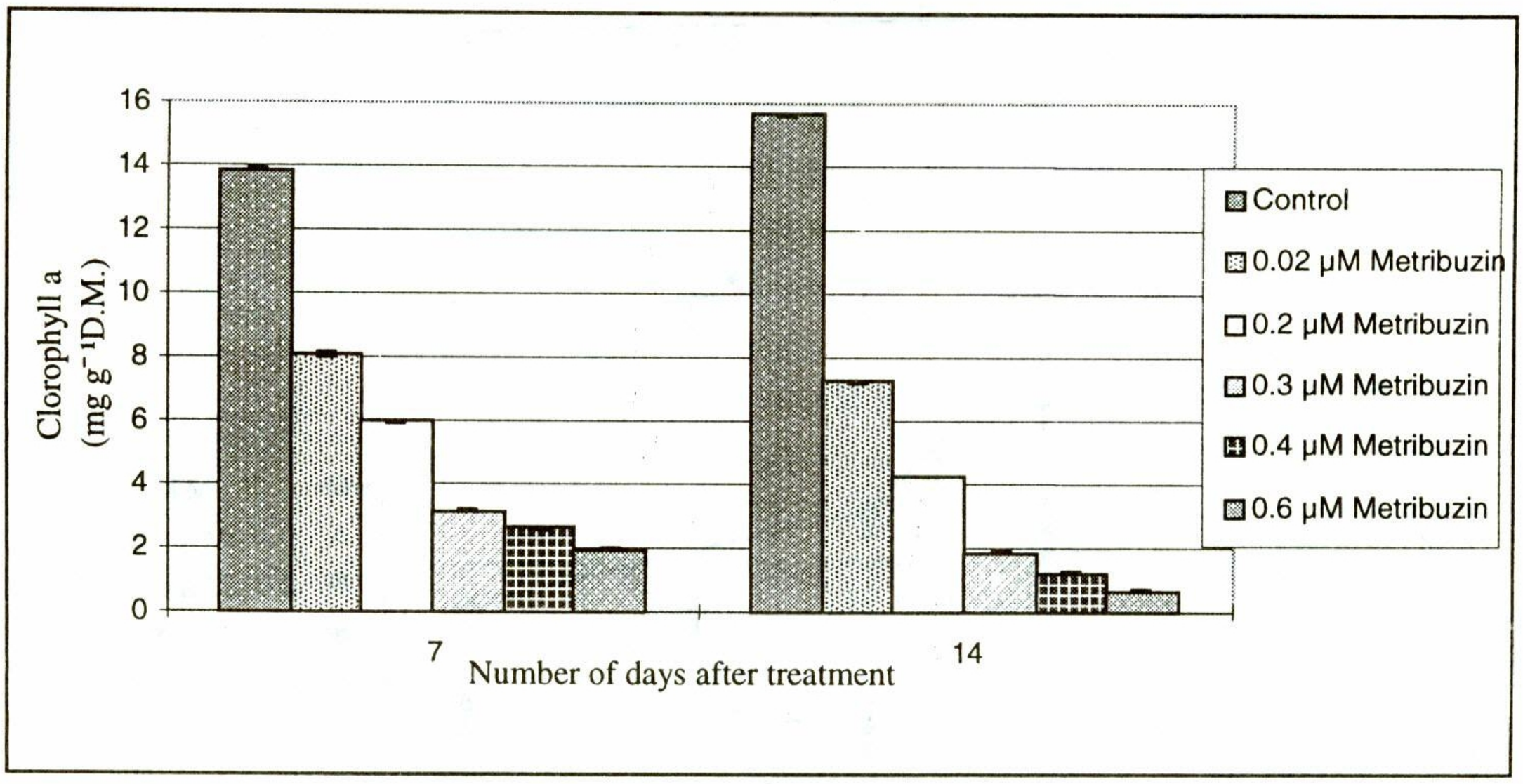


Fig. 5a – The influence of metribuzin on chlorophyll *a* content on *Botryococcus braunii.*

Even the 0.02 µM metribuzin induced after 7 days a significant decrease both in chlorophyllian and in carotenoid pigments. The difference from control became larger in the second week. The increase in control values after 14 days from the beginning of the experiment may be explained as an effect of increasing self-

shading of the *Botryococcus* cells. Assimilatory pigments content was decreased by 0.2 and 0.4 μM metribuzin below 50% and respectively 25% of the control levels (Fig. 5 a, b, c).

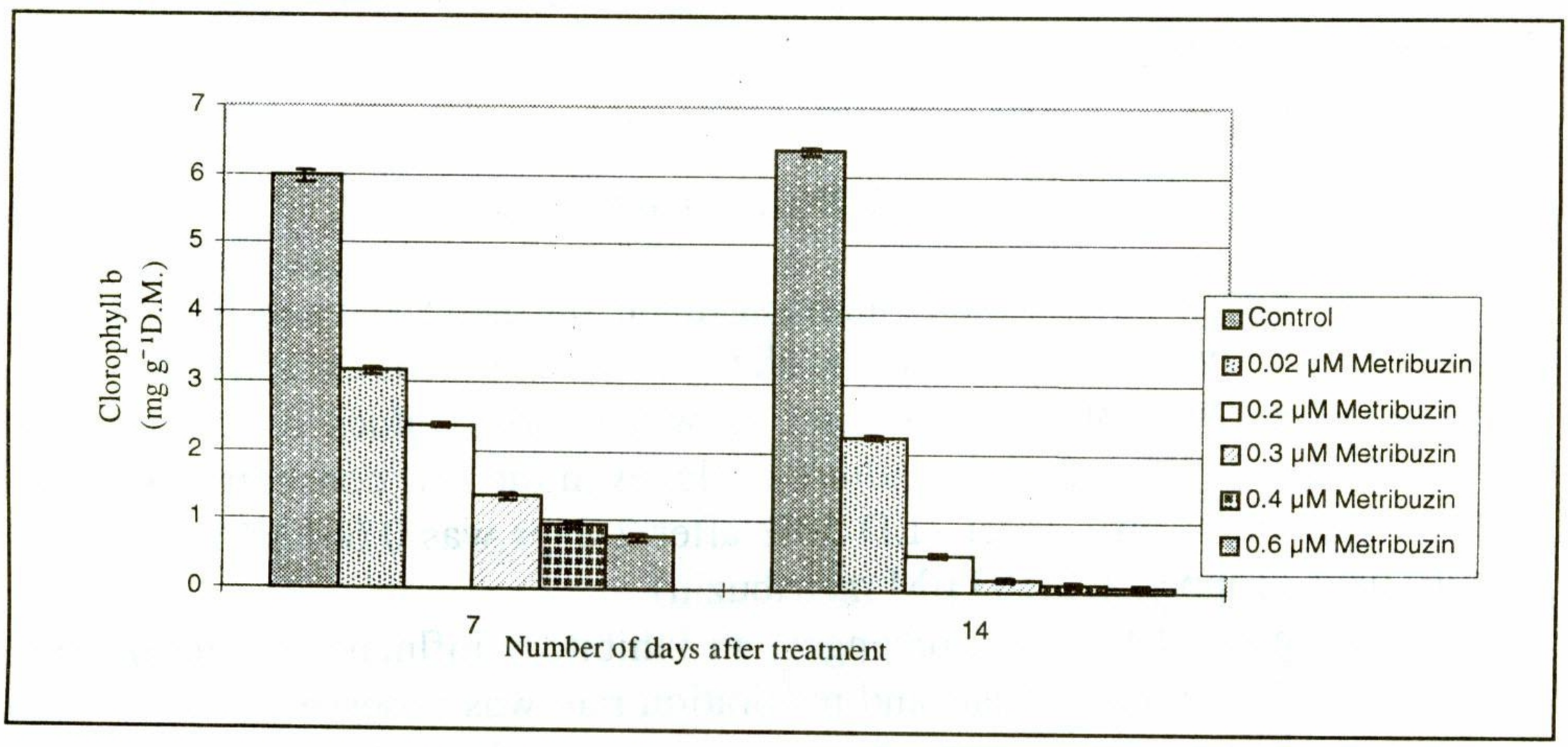


Fig. 5b – The influence of metribuzin on chlorophyll *b* content on *Botryococcus braunii.*

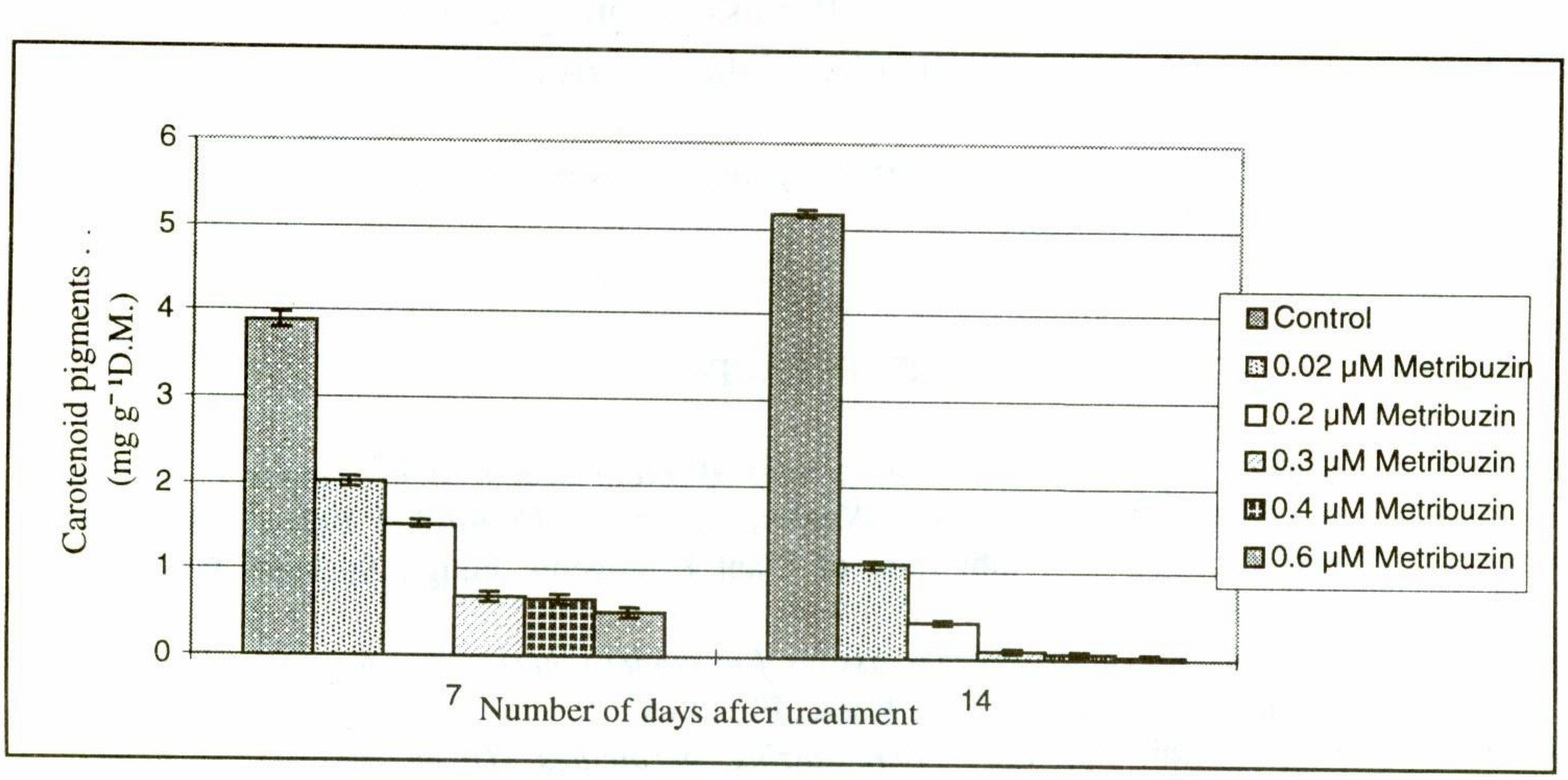


Fig. 5c – The influence of metribuzin on carotenoid pigments content on *Botryococcus braunii.*

This experiment showed the inhibitory influence of the metribuzin herbicide on the growth of *Botryococcus braunii*. In the first growth period, for low concentrations (0.02 μM) the obtained values were relatively close to those obtained for the control. But soon, an obvious depression appeared in the evolution of the growth and of the cell numbers from the algal suspension at all used concentrations.

The metribuzin herbicide in all used concentrations had an inhibitory effect on the other studied physiological processes, too. This proves that the inhibitory action of the herbicides influences all the physiological processes of the plants.

The *Botryococcus braunii* represent a useful model for the study of the herbicide impact on plant physiology.

## CONCLUSIONS

1. The metribuzin herbicide used in this experiment showed that the growth of *Botryococcus braunii* was affected at all used concentrations excepting the 0.02 μM treatment which presented, in the first growth period, values relatively close to control. In the last period of the culture cycle, even the low concentrations showed values below the control level. (LD 50% after 2 days was 0.661 μM and LD 50% after 14 days dropped at 0.163 μM metribuzin).

2. In regard of the gas exchanges, an inhibitory influence of the metribuzin herbicide on the photosynthesis and respiration rate was observed. On the $14^{th}$ day of the experiment, an obvious depression appeared in the evolution of both investigated phenomena.

3. For the 0.2 μM concentration, the assimilatory pigment content showed a decrease below 50% of control value after 7 days of treatment.

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# IDENTIFICATION OF HALOPHILIC *ARCHAEBACTERIA* ISOLATED FROM SOME ROMANIAN SALT LAKES ON THE BASIS OF LIPIDS COMPOSITION

MĂDĂLIN ENACHE, GABRIELA TEODOSIU, ANA-MARIA FAGHI, LUCIA DUMITRU

A relatively large number of *Archaebacteria* belonging to *Natronococcus* (one strain), *Haloarcula* (two strains), *Halobacterium* (three strains), *Halococcus* (four strains) and *Haloferax* (eight strains) were isolated from different salt lakes in Romania with a salt concentration ranging from 60 g/l to 254 g/l. The genera identification was made through lipid composition determination. Unlike the case of usual media, here *Natronococcus* does not show any pigmentation; on the lipid determination medium the pigmentation turns red.

*Key words*: *Archaebacteria*, extreme halophiles, phospholipids, glycolipids, thin-layer chromatography.

## INTRODUCTION

Until recently the members of the extremely halophilic archaebacteria have been regarded as a retired conservative group. The reason is that they were isolated from similar environments using enriched media (5). The analyses of the 16S rRNA and of the lipid composition suggesting its great diversity in extreme halophiles lead to a new and different approach (5,6,9).

Lipids from extremely halophilic bacteria are divided in polar lipids, which are derived from archaeol, and they are soluble in acetone and nonpolar lipids, which are nonsoluble. The archaeol is obtained through the substitution of H atoms in OH group, from the second and the third carbon atoms with the phytanyl group. Polar lipids include phospholipids and glycolipids. In extremely halophilic bacteria there were identified PG, PGS, PGP as phospholipids and S-TGA-1, TGA-2, DGA-1, S-DGA-1, TGA-1 as glycolipids (9,12).

The composition of polar lipid of extreme halophiles seems to be correlated with their taxonomic classification thus leading us to the following genera listing: *Halobacterium, Haloarcula, Haloferax, Halococcus, Natronobacterium* and *Natronococcus* (9, 10, 11).

Unlike the common acyl-ester structure, the alkyl-ether structure of lipids from halophilic bacteria should offer stability to the lipids over the wide range of pH encountered by these bacteria.

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The saturated alkyl chains would confer stability towards oxidative degradation, particularly in those extreme halophiles that are exposed to air and sunlight. In this case polar headgroup sulfate of S-TGA-1 and the phosphate groups of PGP-Me would serve to transport the protons transducer by light-activated bacteriorhodopsin across the outer surface of the purple membrane (11).

In this paper we present the identification of some extremely halophilic *Archaebacteria* isolated from several Romanian salt lakes, located in Prahova county and from Techirghiol lake neighbouring the Black Sea, identified through the composition of the polar lipids.

## MATERIALS AND METHODS

*Cultures and media*: The strains of investigated *Archaebacteria* were isolated from the following lakes: Techirghiol, Telega, Baia Verde, Baia Roşie, Baia Baciului and Grota Miresei. The Techirghiol Lake is situated near the Black Sea while the other four are continental lakes from Prahova county. The strains were isolated and grown in a medium with the following composition (g/l): NaCl-125, $MgCl_2 \cdot 7H_2O$-160, $K_2SO_4$-5, $CaCl_2 \cdot 2H_2O$-0.1, yeast extract-1, peptone-1, soluble starch-1. The medium pH was 7.0–7.2 and the cells were grown at 28°C in a rotary shaker-incubator until they reached the stationary phase.

*Differentiation of Archaebacteria from Eubacteria*: in order to differentiate the strains they were cultivated on the above solidified medium with taurocholic acid added in a concentration of 0.025% (3). The cultures were incubated in these conditions for 10 days at 37°C.

*Determination of salt concentration*: was made by the Mohr method (1).

*Isolation of membrane lipids*: cells of extreme halophiles grew in the above described medium, they were harvested by centrifugation for 15 minutes at 7000 rpm and the pellet was washed with a basic salt solution 4M (12). The washed cells were resuspended in 20 ml of basic salt, to which methanol and chloroform (2/1 v/v) were added and stirred for 1 hour at room temperature. Then the mixture was centrifuged for 5 minutes at 5000 rpm and the supernatant extract was transferred to separator funnels and chloroform-water (1/1 v/v) was added. By being gently shaken the mixture started the separation into two phases. The residue was immediately dissolved in chloroform and would represent the total lipids extract (5, 8, 12).

*Identification of membrane lipids*: thin-layer chromatography (TLC) of the total lipids extract was performed on precoated silica gel 60 plates (Merck). The used solvent system was: chloroform-methanol-acetic acid-water (85/22.5/10/4 v/v/v/v). Phospholipids were detected by spraying with ammonium-phosphomolybdate acid 10% in ethanol in the developed plates. The phospholipids appeared as blue spots after being heated at 150°C (14). Plates were sprayed with

0.5% α-naphthol in methanol-water 1/1 (v/v), dried at 150°C, sprayed with sulfuric acid – water 1/1(v/v) and heated at 150°C for 15 minutes, for detection of glycolipids which appeared as purple spots.

*Identification of genera of the archaebacteria investigated*: was made by comparing the $R_f$ values from this study of glycolipids with $R_f$ values from literature (10, 12).

## RESULTS AND DISCUSSION

*Differentiation of Archaebacteria from Eubacteria*: Bile acids and its salt are able to lyse the cells of halophilic archaebacteria (3,4) while eubacteria cells are not affected. In this way out of 30 strains tested we obtained 16 strains unable to grow with taurocholic acid and thus we assumed that they are *Archaebacteria.* The others 14 belong to *Eubacteria* because they were not inhibited. (Table 1). We noticed that the number of *Archaebacteria* increased in the investigated environments as the salt concentrations increased. Thus, in Baia Verde Lake from five strains isolated only four were *Archaebacteria*. In the Telega Lake, where the salt concentration is 161 g/l (Table 1) out of eight isolated strains five were *Archaebacteria* and in Grota Miresei Lake, with a 254 g/l salt concentration, *Eubacteria* were not detected, while all the three isolated strains were *Archaebacteria* (Table 1). As Table 1 shows the number of *Archaebacteria* isolated from different sources grow with the increase of the salt concentration, while *Eubacteria* is under a simultaneous decrease. These results are similar to other papers (2,13) because extreme halophilic behavior is rather common to *Archaea* while too rare to *Eubacteria* (7).

*Table 1*

Some chemical properties and number of *Archaebacteria* and *Eubacteria* isolated from the lakes investigated

| **Lakes** | **pH value** | **Chloride concentration (g/l)** | **Number of strains isolated** | ***Archaebacteria*** | ***Eubacteria*** |
|---|---|---|---|---|---|
| Techirghiol[a] | – | 60 | – | 3 | – |
| Baia Roşie | 7.9 | 74.9 | 2 | 2 | 0 |
| Baia Baciului | 8.7 | 97.4 | 12 | 2 | 10 |
| Baia Verde | 9.0 | 138.5 | 5 | 4 | 1 |
| Telega | 8.3 | 161 | 8 | 5 | 3 |
| Grota Miresei | 8.3 | 254.6 | 3 | 3 | 0 |

a = all strains isolated are not analyzed

Other distinctive features of the *Archaebacteria* are the presence of ether-linked lipids, components which are detected in the strains isolated from salt lakes in Romania.

*Identification of membrane lipids and genera of Archaebacteria investigated*: After being separated, lipids were analyzed by TLC and the obtaining results are compared with literature data (Table 2) (12). For TLC analysis of the total lipids extract plates covered with silica gel were used as described in Materials and Methods.

*Table 2*

TLC mobility of polar lipids

| Component | $R_f$ from literature | $R_f$ obtained |
|---|---|---|
| PGS | 0.30 | 0.29–0.31 |
| S-TeGA | 0.04 | 0.05–0.07 |
| S-TGA-1 | 0.13 | 0.13 |
| TGA-1 | 0.15 | 0.17 |
| S-DGA-1 | 0.32 | 0.33–0.34 |
| DGA-1 | 0.51 | 0.50 |
| PGP-Me $C_{20}C_{20}$ | 0.25 | 0.26 |
| PGP-Me $C_{20}C_{25}$ | 0.28 | 0.28 |
| PG $C_{20}C_{20}$ | 0.77 | 0.79–0.80 |
| PG $C_{20}C_{25}$ | 0.82 | 0.84-0.89 |

PGP-Me = methylated phosphatidyl glycerophosphate, PG = phosphatidyl glycerol, PGS = phosphatidyl glycerosulphate, TGA-1 and S-TGA-1 three glycosyl archaeol and its derived sulphate, S-TeGA = tetraglycosyl archaeol sulphate, DGA-1 and S-DGA-1 = diglycosyl archaeol and derived sulphate.

In accordance with literature data (8,10), in *Haloferax* genera were detected such glycolipids as DGA-1 and S-DGA-1, in *Halococcus* were identified S-TGA-1 and S-DGA-1, but not the DGA-1 or other glycolipids. For *Halobacterium* genera TGA-1 was identified having as reference for comparison the collection strain *Halobacterium halobium* S93. For *Haloarcula* the phospholipid PGS was detected and for *Natronococcus*, PGP-Me. In this latter case the optimum pH was tested as well as the strains development in specific medium for *Natronococcus* sp.

In *Haloferax, Natronococcus, Halobacterium* and *Halococcus* genera PGP-Me and PG were detected as phospholipid. In some strains such as: T2/5, T1/2, Tl6, Gr2 and Gr3, PGP-Me was not detected but PG was present as phospholipid, excepting the strains Gr2 and Gr3, where PGS was identified. Also, where PG was present, occasionally PGP lacked and so did its derivatives.

Upon the lipids analysis from 18 strains of archaebacteria the following genera were identified: *Natronococcus* (one strain), *Haloarcula* (two strains), *Halobacterium* (three strains), *Halococcus* (four strains),and *Haloferax* (eight strains).

*Table 3*

Identification of *Archaebacteria* strains isolated from Romanian salt lakes on the basis of lipids composition

| | PGPMe | PG | PGS | STGA1 | TGA-1 | TGA-2 | STeGA | SDGA1 | DGA-1 | Genera |
|---|---|---|---|---|---|---|---|---|---|---|
| Gr1 | + | - | - | - | - | - | - | + | + | Haloferax |
| Gr2 | - | - | + | - | - | - | - | - | - | Haloarcula |
| Gr3 | - | - | + | - | - | - | - | - | - | Haloarcula |
| Tl1 | + | + | - | - | + | - | - | - | - | Halobacterium |
| Tl2 | - | + | - | - | - | - | - | + | + | Haloferax |
| Tl3 | + | + | - | + | - | - | - | + | - | Halococcus |
| Tl5 | + | + | - | - | - | - | - | + | + | Haloferax |
| Tl6 | + | - | - | - | - | - | - | + | + | Haloferax |
| BV1 | + | + | - | + | - | - | - | + | + | Halococcus |
| BV2 | + | + | - | + | + | - | - | - | + | Halobacterium |
| BV4 | + | + | - | - | - | - | - | + | + | Haloferax |
| BV5 | + | + | - | - | - | - | - | + | - | Halococcus |
| BR1 | + | - | - | - | - | - | - | + | + | Haloferax |
| BR2 | + | - | - | - | - | - | - | - | - | Natronococcus |
| T2/5 | - | + | - | - | - | - | - | + | + | Haloferax |
| T1/2 | - | + | - | + | + | - | + | - | - | Halobacterium |
| T5/3 | + | + | - | - | - | - | - | + | + | Haloferax |
| BB8 | + | - | - | + | - | - | - | + | - | Halococcus |

Gr = strains isolated from Grota Miresei Lake, Tl = Strains isolated from Telega Lake, BV = strains isolated from Baia Verde Lake, BR = strains isolated from Baia Roşie Lake, T = strains isolated from Techirghiol Lake, BB = strains isolated from Baia Baciului Lake. In strains Gr2 and Gr3 phosphatidic acids were detected.

*Table 4*

Diversity of extreme halophilic archaebacteria in environments investigated

| Lakes | *Halobacterium* | *Halococcus* | *Haloferax* | *Haloarcula* | *Natronococcus* |
|---|---|---|---|---|---|
| Techirghiol | 1 | 0 | 2 | 0 | 0 |
| Baia Roşie | 0 | 0 | 1 | 0 | 1 |
| Baia Baciului | 0 | 1 | 0 | 0 | 0 |
| Baia Verde | 1 | 2 | 1 | 0 | 0 |
| Telega | 1 | 1 | 3 | 0 | 0 |
| Grota Miresei | 0 | 0 | 1 | 2 | 0 |

Strains of *Haloferax* genus were detected in all the six lakes investigated except one (Baia Baciului). The strains of *Halobacterium* were present in

Techirghiol, Baia Verde and Telega lakes, *Halococcus* strains were present in Baia Baciului, Baia Verde and Telega lakes while *Haloarcula* strains were detected in Grota Miresei lake (where the salt concentration is very high) and *Natronococcus* strains were found in the Baia Roşie Lake where pH is around 8.0. (Tables 1 and 4).

## CONCLUSIONS

Glycolipid composition analysis could be used as taxonomical criterion of *Archaebacteria* to the level of genera.

The study shows that the number of *Archaebacteria* strains increases as the salt concentration from the environment increases. Thus in Grota Miresei lake where salt concentrations are very high the *Archaebacteria* are solitarily present.

According to glycolipid composition, we identified strains belonging to all known halobacteria genera. Strains of two or three known genera were detected in all investigated lakes proving the diversity of these *Archaebacteria.*

*Haloarcula* was for the majority of the strains found in high salt concentration environments.

One *Natronococcus* strain was isolated and identified in Baia Roşie Lake. This archaebacterium is able to grow in a 9–11 *p*H environment. We cultivated this strain in a 9.5 *p*H medium for *Natronococcus* sp and the development was very good. The most important thing is that while in its own environment *Natronococcus* lacks pigmentation on our lipid determination medium (*p*H 7.0) the strain is pigmented in red. Probably this medium contains components that affected the biosynthesis of pigments.

In the investigated environments the strains of genera *Haloferax*, *Halococcus* and *Halobacterium* prevailed. For the Techirghiol Lake, situated near the Black Sea, the number of isolated strains is far larger and supplementary investigation is necessary.

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# PRESENCE OF MICROFUNGI IN AN APARTMENT AND SOME HYGIENIC ASPECTS

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High humidity and differences of temperature during the year conduct to mould growth, to serious hygienic problem and aesthetical damages. *Cladosporium, Penicillium*, *Aspergillus* and *Alternaria* genera are the most predominant moulds in the apartment both on the walls and in the air. The main sources are: the air, plants or flowers, food, clothes and dust. The treatment and prevention of fungal growth will include the following procedures: condensation must be decreased, building works will be washed with a strong fungicid solution and repainted with a high performance fungicid paint.

*Key words*: fungi, indoor air, indoor surface, moisture, spore count, airborn spores.

## INTRODUCTION

Starting from the idea that fungi are everywhere they are expected to be found both on indoor surfaces or indoor and outdoor air. Romania is situated climatically in the temperate zone so that during frost free periods most indoor fungal spores are provided by outdoor air. In winter the outdoor fungal spore sources are frozen and/or covered with snow. In consequence indoor spores are less in winter than in the rest of the year (11).

In Europe, *Cladosporium, Alternatia, Epicoccum* and *Aureobasidium* spores are dominant in summer and *Penicillium* and *Aspergillus* in winter (8, 10, 13, 14).

The amount of moisture on a surface and in the ambient air humidity control fungal growth suggesting that fungi appear on moist building substrates in a certain succession, but the primary colonizers are *Penicillium* species. Growing on the wet surfaces they develop microcolonies and spores which wither away when the surface dries up helped by human action and air movement. Organic materials such as plant debris are sources of *Aspergillus* species. House plants may also increase the concentration of airborne fungal spores.

As a result, the biodeterioration and discoloration of wall joints and wallpaper caused by mould growth in residential dwellings in Romania represent a major problem. In particular, high humidity and differences in temperature during the year contribute to mould growth. Also, biodeterioration and discoloration due to moulds constitute a serious hygienic problem.

In this paper we have investigated in laboratory fungal spore concentration in a home environment (air and walls) and the possibility to remove and prevent the recurrence of existing growth.

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## MATERIALS AND METHODS

It was selected an apartment made of concrete with two mouldy walls as a result of a high humidity (approximately 65%).

Airborne fungi were isolated with an air sampler situated in the center of the dining room. Air samples were spread on potato dextrose agar (PDA) containing 0.005 (w/v) streptomycine to inhibit bacterial growth. Plates were incubated at 28 °C for 4–7 days.

The surface samples were taken where the mould growth was visible on the walls (approximately 5 square cm) with sterile swabs. Then the swab was shaken in 10 ml of sterile water and spread on PDA and dichloran glycerol medium (4, 9, 12).

For identification, moulds were examined for the morphological and growth characteristics of the colonies, for the characteristics of conidiogenous cells and conidia (1,14).

## RESULTS AND DISCUSSIONS

### Concentration and composition of the airborne fungi

In the air it was found $1.10^2 - 2.10^2$ cfu/$m^2$ and the most frequent genera were: *Cladosporium* (76%), *Penicillium* (21%), *Aspergillus* (11%), *Botrytis* (9%) and *Phoma* (6%) (Table 1).

*Table 1*

Occurrence of fungi on the walls of an apartment

| **Sample** | **The total colony counts (cfu/$m^3$)** | **Genus** |
|---|---|---|
| left to the window | $3.5 \times 10^3$ | *Cladosporium, Botrytis, Penicillium, Aspergillus, Alternaria* |
| under the window | $7 \times 10^3$ | *Cladosporium, Penicillium, Alternaria, Aureobasidium, Fusarium* |
| right to the window (corner) | $1.4 \times 10^4$ | *Cladosporium, Penicillium, Alternaria,Aspergillus, Phoma, Cunninghamella* |
| in the middle of the wall | $3.5 \times 10^3$ | *Cladosporium, Alternaria, Trichoderma, Penicillium* |
| right to the window | $3.5 \times 10^3$ | *Penicillium, Alternaria* |
| control | $1 \times 10^3$ | *Penicillium, Aspergillus* |

### Moulds on the walls

Samples were taken from different places of the North and North-West walls of the dining room (Table 1).

The main deteriorations of the walls are: crusts, powdery area, brown, yellow, red green or dark stains. Their distribution on the walls is irregular and growth depends on humidity, organic matter, air movement and not in the least on organisms.

One of the most prominent moulds in the apartment studied, as in almost all the domestic dwellings, is *Cladosporium*. This genus has a wide distribution and abundance as a result of its ability to adapt on different substrates and to proliferate in nutrient poor conditions.

*Cladosporium* is in our studies the most abundant fungus indoor air and surfaces but is considered as one of the most prelevant indoor air fungi in the damp residences.

*Penicillium* is a very common genus in the apartment both in the air and on the walls. *Penicillium* and *Aspergillus* spores are called indoor fungi. Being considered as primary colonizers *Penicillium* species require the lowest water activity for growth and develop microcolonies on the temporarily wet wall-producing spores which wither away when it dries up. It could be one of the mechanisms, which explain the spreading of the same moulds both in the air and on the wall.

*Penicillium* and *Aspergillus* produce greenish spots on the wall but at the same time act as biodeteriogens by their organic acids which act as chelating agents.

*Alternaria* also appears in all samples except for the control. This genus as well as *Cladosporium* and *Aureobasidium* produce black pigments involved in esthetic damage. Spores provided by these genera have a mucilage layer which helps them to adhere on the surface. Also *Aureobasidium* produces pullulan, a black exopolysaccharide which sometime generates a thin film on the wall.

*Botrytis, Fusarium* and *Trichoderma* come from the outdoors or indoors. Sources of *Botrytis* and *Fusarium* are probably decaying or diseased parts of foliage plants and/or flowers. The main source for *Trichoderma* is wooden window because it is known as a cellulolytic fungus.

Mould growth causes hygienic aspects beside aesthetic ones. *Cladosporium, Penicillium, Alternaria, Aspergillus* are known as allergenic. Thus dwellings are improper for asthmatic people. Spores are transferred from the dining room to other room including kitchen and larder where organic substrates proper for the generation of a new growth will be found.

Moriyama (7) isolated members of the genus *Exophiala* which cause chromomycosis. We did not find them but they could be found in other places.

Also we did not find *Stachybotrys atra* because it is a saprophytic fungus which colonizes cellulose but Pasanen (11) described a case with a heavy contamination of *S. atra* in building materials. Toxins produced by *S. atra* or *Fusarium* (macrocyclic trichothecene) as a result of a contact with the material

contaminated and a good growth in human body can cause symptoms and diseases for the occupants: signs of cold, sore throat, diarrhea, headache, dermatitis or intermittent focal alopecia (2, 3, 5, 6).

The treatment and prevention of fungal growth are necessary to undertake. Condensation and other sources of water in and on the walls must be decreased by improving ventilation, heating and building works. Microbiological infections could be removed with strong fungicide washing solutions and repainting will be done with high performance fungicid paint.

## CONCLUSIONS

1. High fungal spore concentrations with a large variability and wide spectra of fungi are originated from wet walls.
2. Walls have to be cleaned and protected by building works against water holding back.
3. Preventive measures and providing of improved ventilation, extraction and heating are very important to eliminate the cause of fungal growth.
4. Inhalation of airborne fungal spores is dangerous for the occupants.
5. After cleaning and applying a strong biocide the apartment will be monitored for fungal growth.

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*COMPTE RENDU*

*Enzymology of Disturbed Soils,* Part 26, *Developments in Soil Sciences*, edited by S. Kiss, D. Paşca, M.Drăgan-Bularda, Elsevier, 1998, 336 p.

This volume focuses on one of the phenomena of environmental pollution, namely the disturbance of soils, and represents an updated synthesis of current knowledge on enzyme activities in disturbed soils and their remediation. This topic is reviewed considering both western and eastern literature.

The book is organized in three parts, each part containing well-documented studies reviewed by country, interesting conclusions and many references.

Part I "Enzymology of Oil-contaminated Soils" concentrates on the problems related to the soil enzymological effects of accidental oil contamination, oil contamination of soils and remediation of contaminated soils in experimental models.

The papers grouped under the generic title "Enzymology of Soils Affected by Industrial Emission" are included in Part II of this book and focused on the following aspects: soils enzymological effects of the components of industrial emission through experimentals models in the laboratory or *in situ* artificial microcosms; soil enzymological effects of industrial emission originating from a point source; and soil enzymological effects of industrial emission originating from multiple sources. These aspects make possible to establish the responsibilities of industries in environmental pollution and to evaluate the efficiency of the technologies applied for decontamination.

The last part summarizes the data on "Enzymology of Technogenic Soils", classified according to the nature of the raw material which is used by the industrial technologies causing the wastes accumulation. Enzymological aspects of recultivation, regeneration and transformation of soils disturbed by different industrial activities into agricultural soils are also described.

The volume is supplemented with a Subject index, an Index of the Scientific Names of Microorganisms, Plants and Animals, and an Index of Geographic Names.

In conclusion, this book is an excellent review and an invaluable source of information and ideas for both researchers in specific areas and students.

*Anca Roşeanu*

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# AVIS AUX COLLABORATEURS

La «Revue roumaine de biologie – Série de biologie végétale» publie des articles originaux dans les domaines suivants de la biologie végétale: biologie moléculaire, cytologie, cytogénétique, morphologie, physiologie, génétique, microbiologie, systématique, chorologie, géobotanique, écologie végétale et phytopathologie. Le sommaire est complété par les rubriques: 1. La vie scientifique, qui traite des manifestations scientifiques dans le domaine de la biologie (symposiums, conférences, etc.); 2. Comptes rendus des plus récentes parutions dans la littérature.

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Les textes ne doivent pas dépasser 10 pages (y compris les tableaux, la bibliographie et l'explication des figures).

La responsabilité pour le contenu des articles revient exclusivement aux auteurs.

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# TRAVAUX PARUS AUX ÉDITIONS DE L'ACADÉMIE ROUMAINE

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