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The use of botanical microtechnique paraffin in anatomical studies of lichens

Zastosowanie mikrotechniki parafinowej w badaniach anatomicznych porostów

SUMMARY

The paper describes a simple method of making paraffin slides from dry and unfixed lichen specimens, several methods of their staining and assessment of color stability of used dyes. It was demonstrated for the first time that lichen sections from permanent slides can be hydrated and their structure characteristic of fresh material can be restored.

STRESZCZENIE

W pracy opisano prostą metodę wykonywania preparatów parafinowych z suchych i nieutrwalonych porostów. Omówiono niektóre sposoby barwienia skrawków i oceniono trwałość użytych barwników. Pokazano po raz pierwszy, że skrawki porostów z preparatów trwałych można uwodnić i przywrócić im strukturę właściwą świeżemu materiałowi.

Keywords: microtechnique paraffin, microscopic slides, fruticulous lichens: *Evernia prunastri*, *Ramalina fastigiata*, *Sulcaria sulcata*, *Usnea florida*, *U. subfloridana*

INTRODUCTION

For simple examinations of lichen structure, it is enough to have unstained, hand-cut sections made with a safety razor blade and embedded in glycerogelatine. Because of diminutive size of most of the examined objects, detailed observations of lichens are conducted e.g. on microtome slides from the material embedded in synthetic resins: Nowak, Tobolewski (21), Motyka J. (18), Giordani et al. (12), on the sections from frozen material: Wetmore (23), Brodo and Hawksworth (9), on thallus segments collected with special methods: Grube (13), and also on ultrathin sections using the transmission electron microscope: Asta et al. (5) or the scanning electron microscope: Souza-Egipsy et al. (22), Giordani et al. (12).

The paraffin slides technique, widely used for most biological objects, was not recommended for lichens because they crumbled and fell out of the blocks: Gerlach (11). Despite the fact that many lichenologists used paraffin slides, the publications do not present the details of how these were prepared.

This article presents the methodology of preparing permanent paraffin slides, which we tested on dry, herbarium specimens of lichens. We used several ways of staining sections and showed their color stability on slides used for many years. We also successfully attempted to re-hydrate thallus sections from a permanent microscopic slide.

MATERIAL AND METHODS

The material for investigation consisted of lichen species found in Poland: *Evernia prunastri* (L.) Ach., *Ramalina fastigiata* (Liljebl.) Ach., *Usnea florida* (L.) Wigg., *U. subfloridana* Stirt. and S*ulcaria sulcata* (Lèv.) Bystr. in Brodo et Hawksw. – a species, which grows in Japan (Mts Fuji *ad ramos arbores*). Samples for investigations were taken from the herbarium specimens kept in the University of Białystok Institute of Biology, and from the herbarium of the Department of Botany and Mycology (LBL-L) Maria Curie-Skłodowska University in Lublin.

Softening of material

Fragments of dry thalli were placed for 24 hours in a solution of glycerol, 96% ethyl alcohol and distilled water (G.A.W.) at the ratio of 1:1:1 in order to obtain the steady swelling of lichens (Asahina 4). Thalli can be stored for a longer period in this solution.

A. Preparation of paraffin slides from dry thalli and apothecia of lichens

The slides were made using two methods: 1) classical microtechnique, used in the anatomical examinations of plants, and 2) the simplified method, which does not require dehydration of the material and gradual saturation with paraffin.

1. The paraffin method (classical)

a) Dehydration of material. The material, preserved and swollen in the G.A.W. solution, was dehydrated in an ascending ethanol series at every 10% (from 10% to 96%, each change for 30 min.), then in mixture of absolute ethanol and xylene at the ratios of 2:1, 1:1, 1:2 (for 30 min.), and next in pure xylene three times (1 x 45 min., 2 x 60 min.).

b) Saturation with paraffin (paraffin with melting temp. of 56–58°C was used).

The dehydrated material was placed on cold paraffin in small evaporating dish, poured with several xylene drops and placed in a thermostat at ca. 65°C. After the paraffin melted (after ca. 2 hours) and material precipitated to the bottom of the vessel, paraffin was replaced by a pure compound twice (each change for 1 hour).

c) Preparation of paraffin blocks. A preheated metal mold, covered with glycerol inside, was filled with hot paraffin and the material from the evaporating dish was transferred into it, using a heated metal spoon. Then the mold with paraffin was slowly immersed in water with ice and a paraffin block was taken out from the mold after 15 min. Pyramid-shaped small pieces with material were then cut out from it.

d) Preparation of sections. A paraffin pyramid with material to be cut was fixed with a hot scalpel to the microtome base. Apothecia were cut transversely. The way of cutting paraffin-embedded thalli is shown by dashed lines in Tab. I (Fig. 1, 2, 3, 4). The material was cut with a rotatory microtome into sections ca. 15 μm thick (with thinner sections the algae layer was damaged). The sections were transferred into a drop of water on the slides covered with a binding agent (chicken egg white) and heated until they straightened. The slides were dried in a dryer at the temperature of 36°C.

2. The paraffin method (simplified)

Softened material was used (as above).

a) Embedding in paraffin. Small thallus fragments or apothecia taken out from the G.A.W. solution were dried on filter paper and transferred with a preparation needle directly onto the paraffin pyramid (paraffin melting temp. at 56–58°C). Paraffin was slightly melted with the hot needle and the material was suitably positioned. After the paraffin congealed (several minutes of cooling in the refrigerator is enough) the pyramid was cut in such a way that the material was embedded in as little paraffin as possible but sufficient to hold the material during cutting. In this method material is only slightly saturated with paraffin.

b) Preparation of sections – as in 1d).

B. Staining of slides

Staining was done on sections obtained with the above two methods. Before staining, the slides were deparaffinated, hydrated in a descending alcohol series (from absolute alcohol to 10%) and transferred to water. Several well-known methods were used for staining of sections: 1) Ehrlich hematoxylin (Gerlach, 11), 2) Alum-glycerol hematein according to Rawitz (Gerlach 11), 3) PAS method (Broda 8). All slides after staining were dehydrated in a standard way (ethanol, xylene) and embedded in Canada balsam. Two staining methods described below were also used, which were not applied to lichens before. 4) Staining of mycelia in plant tissues according to Pianese (Bagiński 6): Hydrated sections were stained for 15–45 min. in the solution composed of: Martius yellow (0.01 g); malachite green (0.5 g); acid fuchsin (0.1 g); ethanol 25% (200 ml). They were washed in distilled water for 1 min. and differentiated in a solution containing 0.5% HCl in 95% ethanol. The slides were transferred to absolute ethanol for 5 min. and then made transparent in a solution containing 40 g of phenol dissolved in 60 ml of turpentine. They were transferred into xylene (2 x 5 min) and embedded in the balsam. 5) Staining for detection of mycelia in plant tissues (Bagiński 6): Hydrated sections were stained for 1 hour in a 0.1% thionin solution in 5% carbolic water (aqueous solution of phenol). They were dehydrated quickly, starting with 70% ethanol to absolute ethanol. They were differentiated in saturated (ca. 0.3%) orange G in absolute ethanol, washed in absolute alcohol, transferred into xylene and embedded in balsam.

C. Hydration of permanent slides

The sections prepared with the paraffin technique are somewhat shrunk because of dehydration in alcohol. We checked twice if they were capable of being rehydrated. First we made tests on the sections already prepared for embedding in Canada balsam, transferring them from xylene, through an alcohol series, into water. The sections straightened to the form that fresh moist material has.

Ten years after permanent slides were made from the thallus of *Usnea subfloridana,* embedded in Canada balsam, we attempted to rehydrate them. Before this procedure, the sections on the slides were photographed and their position on the glass was recorded. The slides were then placed

in xylene overnight in order to remove cover glasses. The slides were subsequently hydrated in an alcohol series, washed in water and embedded in 50% glycerol. The same sections were photographed again.

All slides were examined with Nikon Eclipse E 600 microscope. Microphotos were recorded on Kodak film.

RESULTS

The softening of thalli and apothecia in the G.A.W. solution as the preliminary stage before the paraffin technique had a favourable effect on the cutting of material in both methods employed.

RESULTS OF THE STAINING OF LICHENS

The specifications of different ways of staining lichens are shown in Tables II–VI.

1. Lichens stained with Ehrlich hematoxylin:

Usnea subfloridana (Tab. II – Fig. 1a, 1b, 1c; Tab. VI – Fig. 1, 1a, 1b), *Sulcaria sulcata* (Tab. III – Fig. 1a–d; Tab. V - Fig. 2), *Evernia prunastri* (Tab. IV – Fig. 1), *Usnea florida* (Tab. V – Fig. 1, 1a)

Staining result: cortex of the species examined is permanently stained violet, it rarely fades or turns violet-beige (e.g. Tab. II), medulla and axis (*Sulcaria sulcata, Usnea florida* and *U. subfrloridana*) – fungal hyphae pale violet, apothecium (*Usnea florida*, *Sulcaria sulcata*) – bright violet, structure clearly visible (Tab. V); photobiont cells – not stained in this method, preserve their natural green colour on all slides, even many-year-old ones.

2. Lichens stained with alum-glycerol hematein according to Rawitz:: *Usnea subfloridana* (Tab. II – Fig. 2a, 2b, 2c), *Usnea florida* (Tab. V – Fig. 4), *Ramalina fastigiata* (Tab. V – Fig. 5). *Sulcaria sulcata* (Tab. III – Fig. 2).

Staining result: cortex of the species examined violet-pink, stable color, fading with age; medulla – hyphae bright violet; axis (*Usnea* and *Sulcaria*) – fungal hyphae bright violet, axial structure clear; apothecium (*Usnea subfloridana*, *Ramalina fastigiata*) individual layers differ in color intensity (Tab. V); photobiont cells – not stained in this method, retain their natural color on all slides, even many-year-old ones.

3. Lichens stained with PAS method: *Sulcaria sulcata* (Tab. III – Fig. 3).

Staining result: pink staining of cortex and medulla indicates polysaccharidic nature of exocellular matrix (cell walls and binding substances) on fungal hyphae.

4. Lichens stained for detection of mycelia according to Pianese: *Usnea subfloridana* (Tab. II – Fig. 3a, 3b, 3c), *Evernia prunastri* (Tab. IV – Fig. 2), *Ramalina fastigiata* (Tab. IV – Fig. 3; Tab. V – Fig. 3).

Staining result: cortex - in *Evernia prunastri* and *Ramalina fastigiata* grey green; colouring is permanent; in *Usnea* (Tab. II) turquoise-coloured, unstable (considerably fading with age): medulla - in *Evernia and Ramalina* fungal hyphae - grey green; medulla and axis in *Usnea subfloridana* – pink; apothecium in *Ramalina* – asci turquoise-coloured, spores brighter, distinct paraphyses: colouring of asci unstable, photobiont cells – on all slides intensely stained pink-red, colouring stable.

5. Reaction for detection of mycelia in plant tissues (Bagiński 6) was conducted in *Ramalina fastigiata* (Table IV): Staining result: cortex and medulla - fungal hyphae yellow, interhyphal area of cortex (extracellular cementing substances = interhyphal gel) – violet blue. Colour is stable, does not change after many years.

THE EXPERIMENT WITH HYDRATION OF 10-YEAR-OLD PERMANENT SLIDES

Examplary photographs of the experiment are shown in Tab. VI. The test demonstrated that lichen sections from a permanent slide after balsam removal can be gradually hydrated and their size enlarged, while their internal structure becomes more distinct (compare Fig. 1 and Fig.1a). During the process of slide hydration, the cortex was partially decoloured whereas the colour of photobiont cells did not change (Fig. 1a and 1b).

Note: During hydration of slides positioned vertically in the cuvette, many sections became unstuck and came away from the glasses. We recommend that slides be hydrated in a horizontal position and that liquid be changed without removing cover glasses.

DISCUSSION

The classical paraffin method is time-consuming although simple and inexpensive. We demonstrated that permanent good-quality lichen slides can be made with this technique without chemically fixing the material and that the same dyes can be used as for other plant groups. The softening of dry lichens in a G.A.W. solution before embedding them in paraffin had a favourable effect on cutting sections and did not hamper staining. This manner of softening thalli was introduced by Asahina (4), but he did not describe the method of making sections.

Some taxonomists and florist specialists used a simplified version of the paraffin method when identifying lichen species. They embedded dry or water-

soaked material (Motyka M. 19, Bystrek 10, Motyka J. 18), or wetted it with water before embedding it in paraffin and while cutting sections on the microtome (Nowak and Tobolewski 21).

To stain lichens, similar dyes as for plants and fungi can be used, but staining results are not always analogous. Out of the dyes we tested, the universal ones are hematein according to Rawitz and Ehrlich hematoxylin, the dyes which produced violet staining of mycobiont hyphae in the thalli of *Usnea*, *Evernia prunastri* and *Sulcaria sulcata*; in the first two species – especially intensely in the cortical part. On the same slides, the assimilation dyes of photobiont retain natural green colour, even though they were repeatedly washed in alcohol during preparation of slides. We observed green lichen algae also on many-year-old slides. It is commonly known that even on hand-made lichen sections treated with HCl or 10% KOH, algal chlorophyll does not change colour. This shows its high stability as compared with chlorophyll of vascular plants (*Tracheophyta)*. Chlorophyll of lichen algae, like that of autonomous algae, is also more difficult to extract than leaf chlorophyll (Bogorad 9).

Using atypical stains for lichens i.e. for detection of parasite fungi in plant tissues, we found that on slides the hyphae of lichenizing fungi became not stained in the way typical of fungi that infect higher plants, though data in literature show that there are no fundamental differences cytologically between lichenized and non-lichenized fungi (Honegger 14) We believe that the factor that modifies staining results is the changed chemical composition of the cell-wall apoplast of invasive fungi as compared with lichen fungi. Thus, in the method according to Pianese (Bagiński 6) the hyphae of parasite fungi turn dark red, while on our slides the mycobiont hyphae, depending on lichen species, were stained in grey green (*Evernia prunastri* and *Ramalina fastigiata*) or pink (*Usnea subfloridana*). In another method for mycelia detection in plant tissues (also described by Bagiński 6), parasite fungi are stained red-violet, and the cell walls of host plant turn yellow green. After this staining was used in *Ramalina fastigiata*, the chitin hyphae of mycobiont turned yellow (in the way typical of cellulose and pectin walls of higher plants!) whereas the interhyphal apoplast in the cortical part of thallus became blue violet.

On our slides made from lichen thalli the cortex usually became stained the strongest (the cortical apoplastic compartment composed of hyphal walls and substances that bonded them). Owing to the content of appropriate constituents, this outer thallus part makes it easier for lichens to absorb water and the compounds in it. Our observations showed that the cortical part of thallus of *Usnea subfloridana* and asci in hymenium in *Ramalina fastigiata* contain components of similar nature (as the similar result of staining with the Pianese method).

Differences in the reaction of lichen cortex and medulla to the action of reagents can be also demonstrated with simple procedures. In many lichen species

after 10% KOH treatment, the cortical part of thallus (hyphae and interhyphal apoplast) exhibits no colour reactions (e.g. in *Usnea subfloidana*) whereas hyphae of the medulla turn yellow or orange.

PAS method based on insoluble polysaccharides, chitin of fungal hyphae should be clearly coloured in pink to red violet (Broda 8). In lichens of *Sulcaria sulcata* we observed more intense pink staining of the hyphal cell walls and exocellular matrix in medulla than in the upper cortex, in contradistinction to results of PAS test in *Physconia perisidiosa* (Giordani et al. 12). *Sulcaria sulcata* contains quantities for polysaccharides and is used as an edible species in countries of south-eastern Asia (Bystrek 10)

Lichens are characterized by daily periodicity of water absorption and evaporation, depending on ambient temperature and air humidity (Bystrek 10, Matwiejuk 16). Under adverse conditions these organisms can survive long periods of drought after which they do not lose their ability to absorb water. Not only desiccated lichen thalli but, as our experiment showed, also lichen sections from permanent slides retain hygroscopic properties and can regain their original size despite the fact that there are no more living protoplasts in the slides. The mechanism of water absorption by lichens unlike to plants must be independent to high degree of the protoplast (it functions alongside the mechanism engaging the living part of the cell) and comes from: i) the hydroscopicity of the thallus (free water penetrates into the interhalline spaces) and ii) physicochemical properties of the constituents themselves of extracellular gelatinous matrix, which are not destroyed depside being treated with chemical reagents used in the paraffin microtechnique. Detailed investigations on water distribution in foliose lichens were carried out on species from a semi-arid habitat (Souza-Egipsy et al. 22); the showed species-specific differences in anatomy, hygroscopic features of the upper cortex, and differences in the presence and amount of free extracellular water inside the thallus.

Dehydrated sections in permanent slides are somewhat shrunk but they can be stored for a very long time in this condition and serve taxonomists utilizing various collections as comparative material. They can also be used for other purposes. Asahina (1, 2, 3, 4) conducted on anatomical sections his studies on microcrystallography of lichen acids and their location in the thalli of different lichen species.

We should also mention the methods for embedding sections. Glycerogelatine or 50% glycerol does not cause shrinking of thalli and apopthecia but it accelerates fading of slides. We used Canada balsam for permanent slides with good results.

CONCLUSIONS

1. Softening of dry lichens in a solution containing glycerol-ethanol-water (1:1:1) before embedding them in paraffin has a favourable effect on the quality of prepared sections (it reduces material fragility).

2. Slides from lichens become permanently stained with Ehrlich hematoxylin and hematein according to Rawitz. Staining for mycelia detection after Pianese method can be used only for short-term observations. The PAS method does not give distinct results in all lichens.

Lichen fungi become coloured differently from fungi that parasitize in plant tissues.

3. Lichen sections sealed in permanent slides do not lose their hygroscopic properties: after the removal of balsam and hydration they return to the initial form characteristic of fresh material.

4. Permanent slides from lichens are ideal evidence material in taxonomic studies, easy to store.

REFERENCES

1. Asahina Y. 1936. Mikrochemischer Nachweis der Flechtenstoffe, I. J. Japan. Bot. XII, (7): 66–75.
2. Asachina Y. 1936a. Mikrochemischer Nachweis der Flechtenstoffe. II. J. Japan. Bot., XII (12): 25–38.
3. Asachina Y. 1937. Mikrochemischer Nachweis der Flechtenstoffe. III. J. Japan. Bot., 13(7): 59–66.
4. Asahina Y. 1956. Lichens of Japan. Vol. III. Genus *Usnea*. Research Institute for Natural Resources Shinjuku, Tokyo.
5. Asta J., Orry F., Toutain F., Souchier B., Villemin G. 2001. Micromorphological and ultrastructural investigations of the lichen-soil interface. Soil Biol. Bioch. 33: 323–337.
6. Bagiński S. 1969. Technika mikroskopowa. PWN, Warszawa.
7. Bogorad L. 1962. Chlorophylls, in: Lewin R.A. Physiology and Biochemistry of Algae, Academic Press, New York and London.
8. Broda B. 1971. Metody histochemii roślinnej, PZWL, Warszawa.
9. Brodo I. M., Hawksworth L. D. 1977. *Alectoria* and allied genera in North America. Opera Botanica 42: 1–164.
10. Bystrek J. 1997. Podstawy lichenologii. Wydawnictwo UMCS. Lublin.
11. Gerlach D. 1972. Zarys mikrotechniki botanicznej (translated from German). PWRiL. Warszawa.
12. Giordani P., Modenesi P., Tretiach M. 2003. Determinant factors for the formation of the calcium oxalate minerals, weddellite and whewellite, on the structure of foliose lichens. Lichenologist 35(3): 255–270.

13. Grube M. 2001. A simple metod to prepare foliicolous lichens for anatomical and molecular studies. Lichenologist: 23: 547–550.

14. Honegger R. 1996. Mycobionts. In: Lichen Biology (T. H. Nash III, ed.): 23–46. Cambridge. UK: Cambridge University Press

15. Jahns H. M. 1973. Anatomy, morphology and development, In: The Lichens, ed. V. Ahmadijan, Mason E. Hale) Chapter 1: 3–58. Academic Press. New York and London.

16. Letroit-Galinou M. A. 1973. Sexual reproduction, In: The Lichens (ed. V. Ahmadijan, Mason E. Hale) Chapter 2: 59–90. Academic Press. New York and London.

17. Matwiejuk A. 2000. Water content in terricolous lichens, Acta Soc. Bot. Pol. 69(1): 55–63.

18. Motyka J. 1995.Porosty (*Lichenes)* 1. *Lecanora.* Lubelskie Towarzystwo Naukowe.

19. Motyka M. 1969. Hymenial algae in some species of the genus Lecanora (Lichenes). Ann. UMCS Lublin – Polonia, 24 (3): 15–19.

20. Nienburg W. 1926. Anatomie der Flechten (In: Handbuch der Pflanzenanatomie (Linsbauer K., ed.,) 6.2, 1: 1–136 Brontraeger, Berlin.

21. Nowak J., Tobolewski Z. 1975. Porosty polskie. PWN. Warszawa, Kraków.

22. Souza-Egipsy, Valladares F. Ascaso C. 2000. Water distribution in foliose lichen species: interactions between method of hydration, lichen substances and thallus anatomy. Ann. Bot. 86: 595–601.

23. Wetmore C. M. 1960. The lichen genus *Nephroma* in North and Middle America. Publ. Museum, Michigan State Univ. Biol. Ser. 3(4): 209–464.

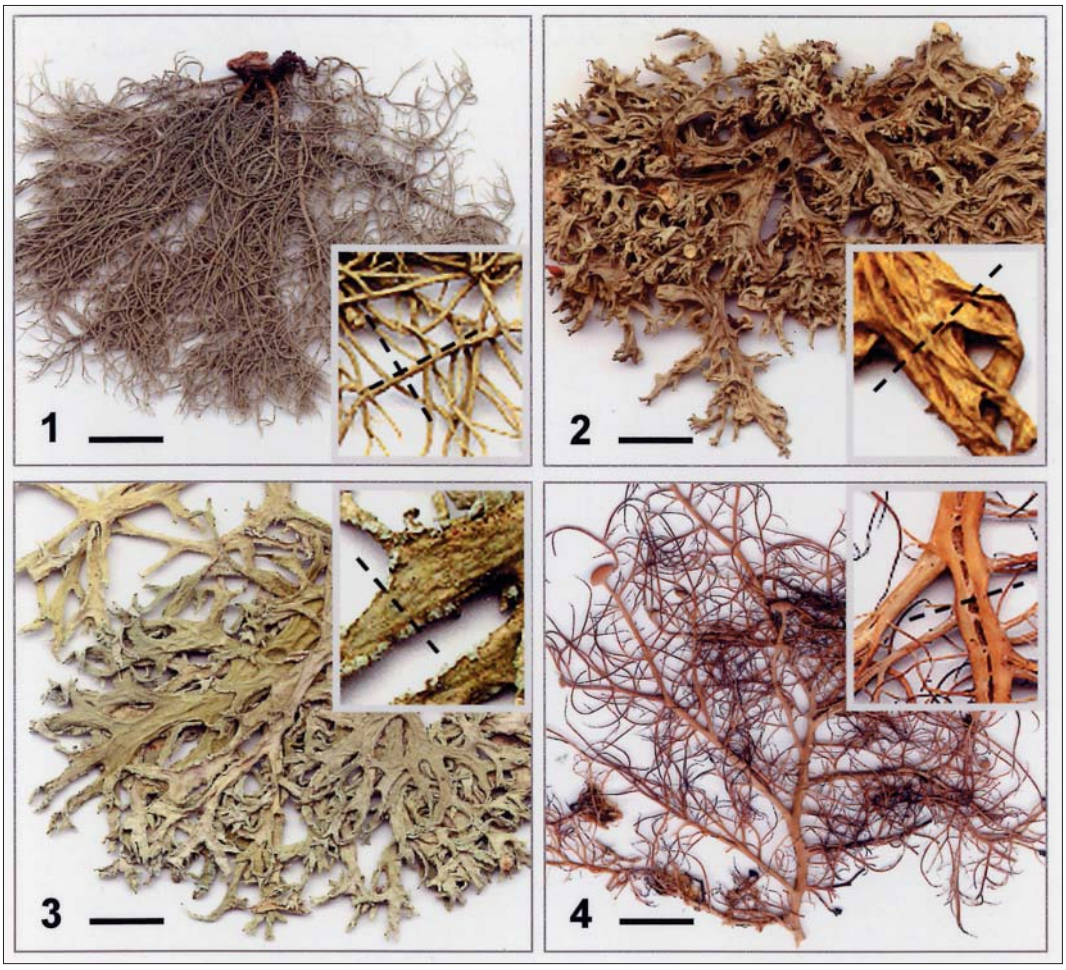

Table 1. The herbarium specimens of lichens investigated: Fig. 1 – *Usnea subfloridana* Stirt.; Fig. 2 – *Ramalina fastigiata* (Liljebl.) Ach.; Fig. 3 – *Evernia prunastri* (L.) Ach.; inserts in Figs. 1–3 – thallus segment, magnified 3x; Fig. 4 – *Sulcaria sulcata* (Lèv.) Bystr. in Brodo et Hawksw., insert: segment of nether part of main branch, magnified 3x. Dashed lines show the manner of cutting thallus while preparing sections. Bar = 1 cm

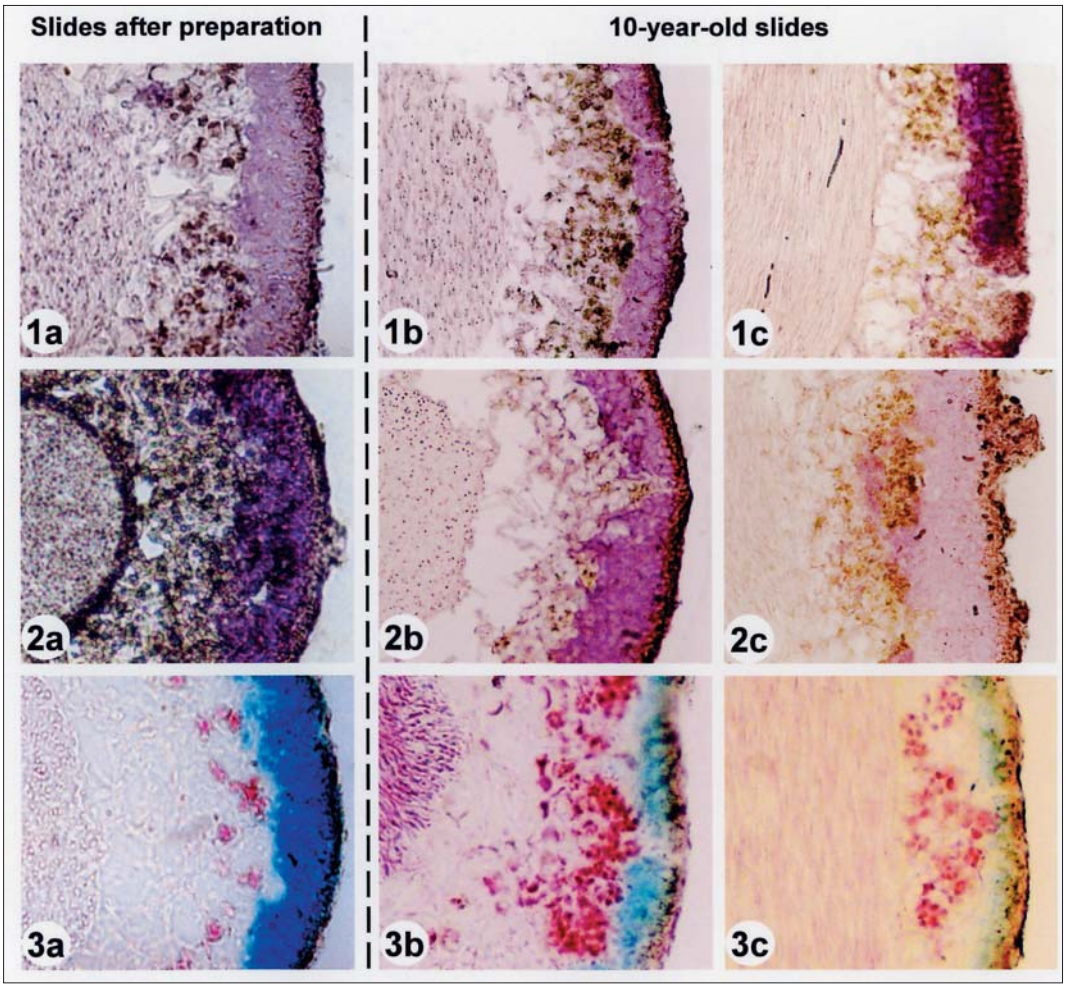

Table 2. Different ways of staining thallus of *Usnea subfloridana* Stirt. Fig. 1 a–c – Ehrlich hematoxylin; Fig. 2 a–c – Alum-glycerol hematein according to Rawitz; Fig. 3 a–c – Staining of mycelia according to Pianese. Figs. a, b – transverse section of branch; Fig. c – longitudinal cross-section of branch; all Figs. x 200

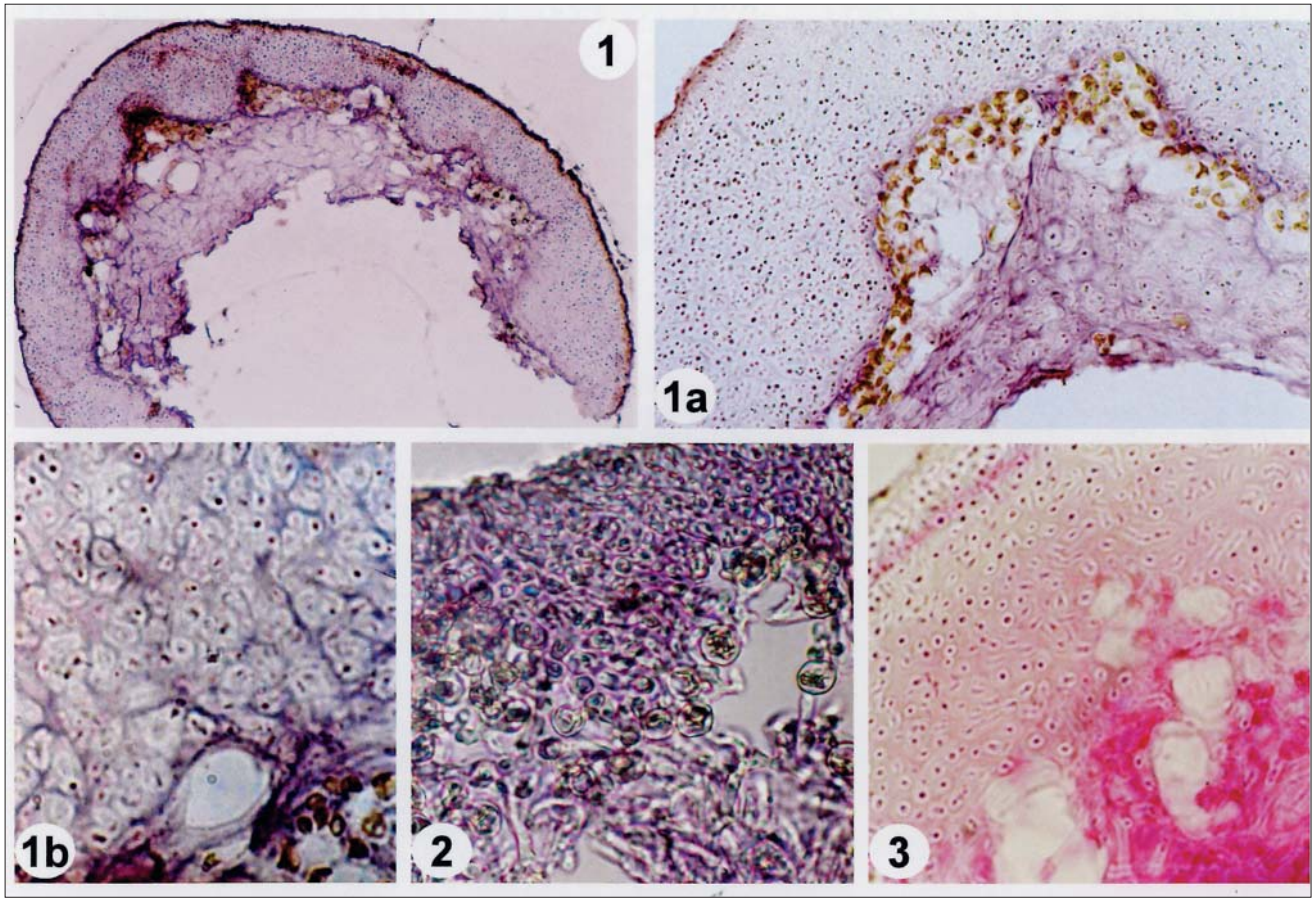

Table 3. Different ways of staining thallus of *Sulcaria sulcata* (Lèv.) Bystr. in Brodo et Hawksw.; all Figs.: transverse section of branch. Figs. 1, 1a, 1b – Ehrlich hematoxylin; Fig. 1 – general view, x 125; Fig. 1a – cortex segment with algal layer and medulla, x 250; Figs. 1, 1a – 10-year-old slides; Fig. 1b cortex segment with algae; slide after preparation x 500; Fig. 2 – hematein according to Rawitz; cortex segment with algae and medulla, slide after preparation, x 500; Fig. 3 – PAS test, pink staining indicates polysaccharidic nature of mycobiont apoplastic compartment in cortex and medulla, 10-year-old slide, x 500

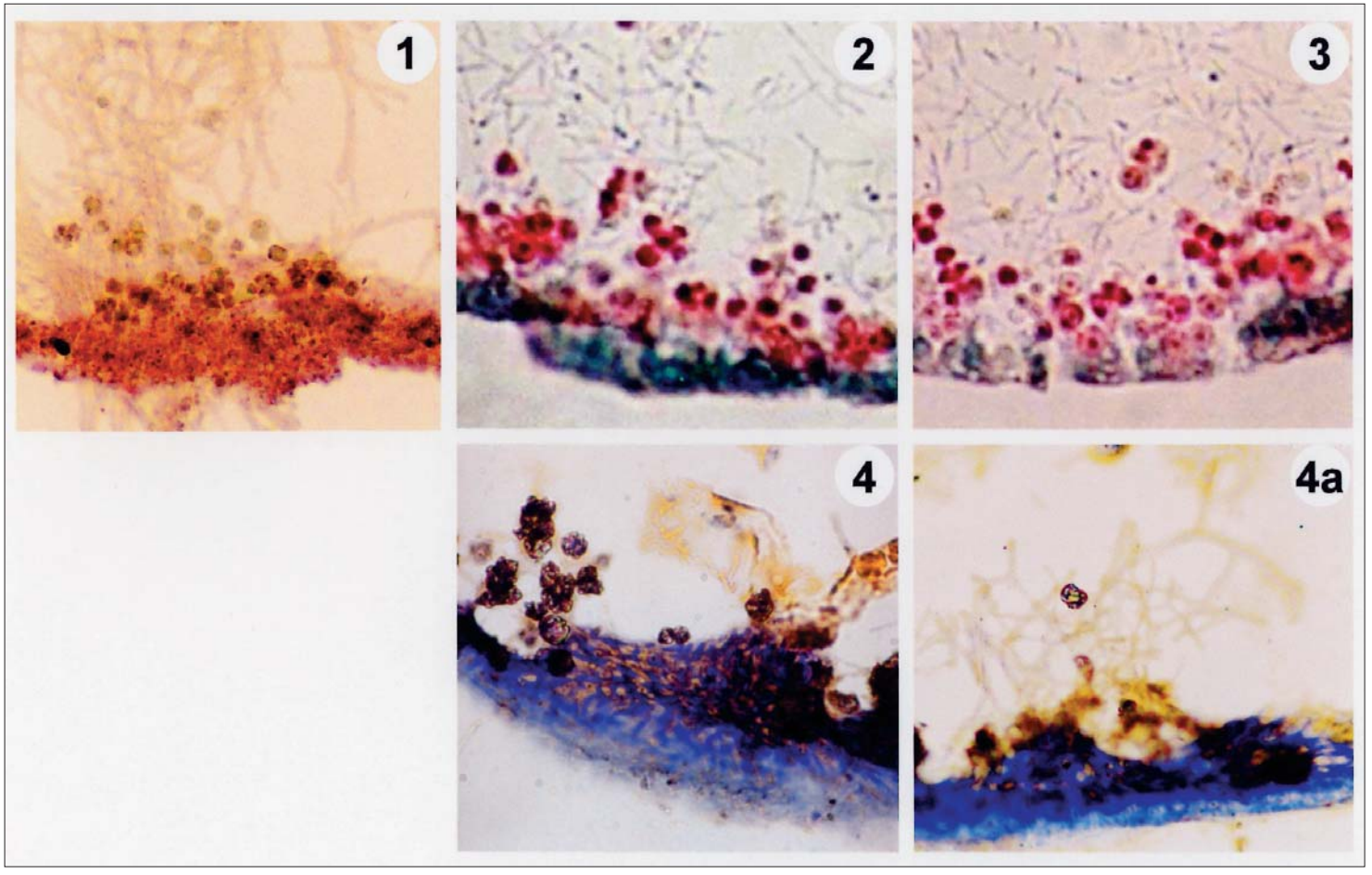

Table 4. Different ways of staining of lichens; all Figs.: transverse section of upper part of thallus, x 300; Fig. 1 – *Evernia prunastri* (L.) Ach., Ehrlich hematoxylin; Fig. 2 – *Evernia prunastri* (L.) Ach.; staining of mycelia according to Pianese; Fig. 3 – *Ramalina fastigiata* (Liljebl.) Ach.; staining of mycelia according to Pianese; Figs. 1–3 – 10-year-old slides; Figs. 4, 4a – *Ramalina fastigiata* (Liljebl.) Ach., staining for mycelia detection in plant tissues; Fig. 4 – slide after preparation; Fig. 4a – 10-year-old slide

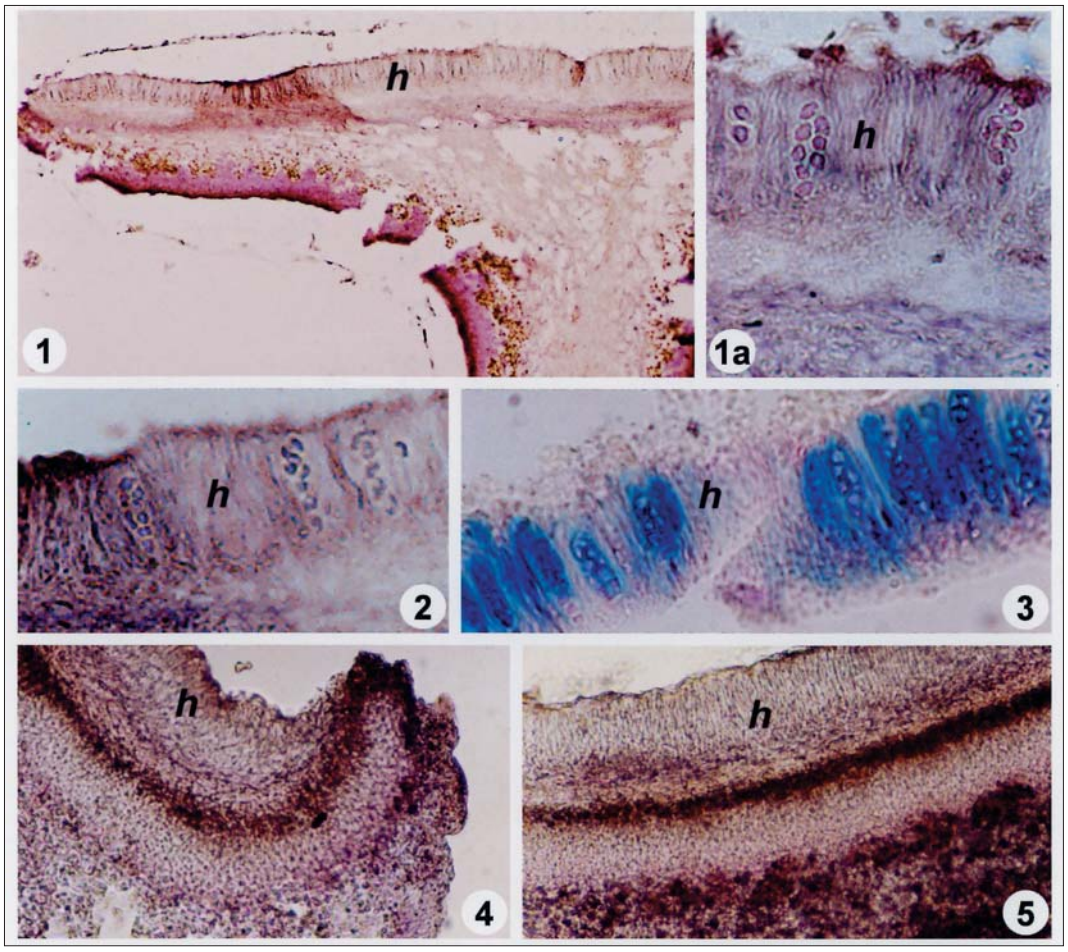

Table 5. Different ways of lichen apothecia staining; *h* – hymenium; Fig. 1 – *Usnea florida* (L.) Wigg., segment of fruiting body with branch; Ehrlich hematoxylin, x 70; Fig. 1a – *Usnea florida* (L.) Wigg., magnified part of fruiting body, Ehrlich hematoxylin, x 450; Fig. 2 – *Sulcaria sulcata*, part of fruiting body, Ehrlich hematoxylin, x 450; Fig. 3 – *Ramalina fastigiata* (Liljebl.) Ach., part of fruiting body, staining according to Pianese; x 450; Fig. 4 – *Usnea florida* (L.) Wigg., portion of fruiting body, staining according to Rawitz, x 140; Fig. 5 – *Ramalina fastigiata* (Liljebl.) Ach., part of fruiting body, staining according to Rawitz; x 140; Figs. 1, 2 – 10-year-old slides, Figs. 1a, 3, 4, 5 – slides after preparation

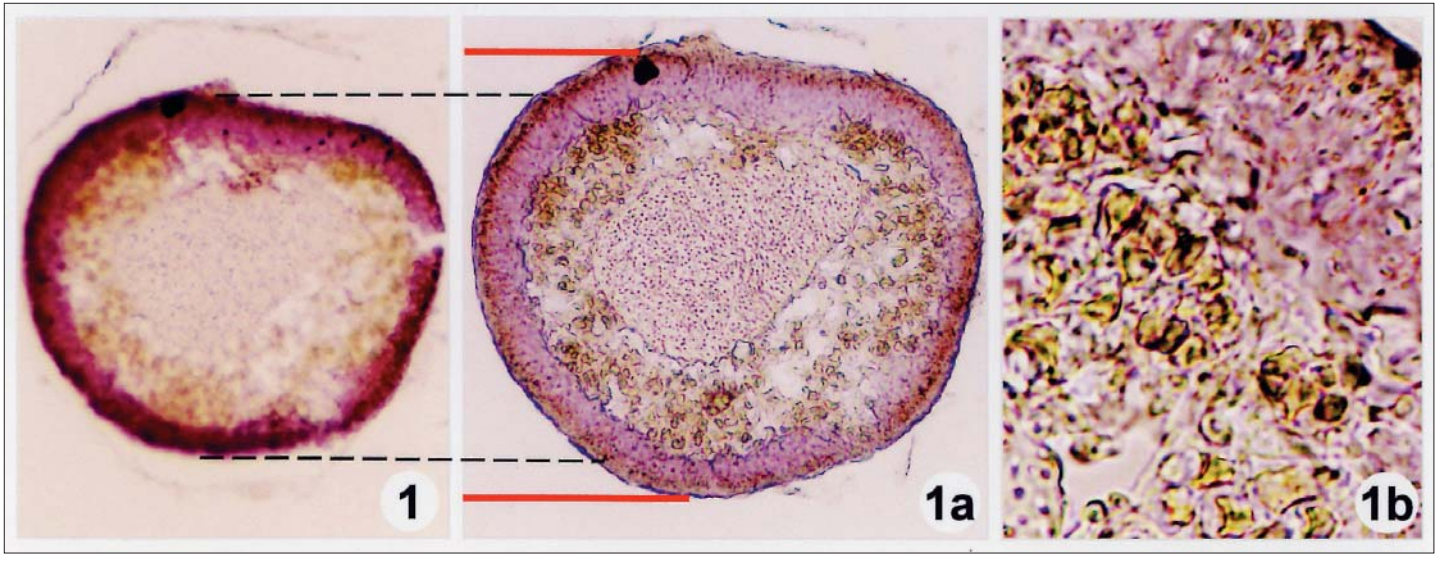

Table 6. Test with hydration of permanent slide, *Usnea subfloridana* Stirt., Ehrlich hematoxylin; Fig. 1 – Section in a 10-year-old slide, x 175; Fig. 1a – the same section as in Fig. 1, but after hydration; red lines show to what size the section became enlarged, x 175; Fig. 1b – Enlarged segment from Fig. 1a, x 700