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VITAL VERSUS HERBARIUM TAXONOMY:

MORPHOLOGICAL DIFFERENCES BETWEEN LIVING AND DEAD CELLS OF ASCOMYCETES, AND THEIR TAXONOMIC IMPLICATIONS [1]

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- in vivo veritas -

ABSTRACT

Micromorphology of fungal cells as observed under the light microscope differs considerably when comparing living with dead cells. Furthermore, different mounting media in current use may highly influence the appearance of fungal cells, and ontogenetic alterations increase the scope of their variability. Taxonomical work must, however, be based on organs in compatible states and development stages. Arguments are presented for diagnoses based on the morphology of living unaltered cells from freshly collected specimens. Living ascocarp cells are to be studied in their fully hydrated state by mounting in aqueous solutions with a very low concentration of ingredients. Tap or rain water is a suitable mountant. Vital taxonomy provides many additional and often new characters of high taxonomic value which are furthermore often more consistent. These can be observed by easy and rapid methods from the living fungus but become obscure or disappear completely in the herbarium. Different kinds of cytoplasmic inclusions are especially concerned. The development stage of a given organ can be much more precisely ascertained in the vital state. Vitality of single cells can be recognized by their

[1]) Based on a poster (IB-67/2) and a video film given at the Fourth International Mycological Congress held in Regensburg, F. R. G., 28th Aug. - 3rd Sept. 1990 (BARAL, 1990).

osmotic response, by the appearance of their cytoplasm, or through staining *in statu vivo* with basic dyes. Striking alterations during the death of fungal cells are, e.g., shrinkage for about 30-50% of the original volume, or expansion of inner wall layers to about 2-5 times their normal thickness. One new combination is proposed: **Allophylaria nervicola** (Velen.) Baral *comb. nov.*

ZUSAMMENFASSUNG

Die Mikromorphologie pilzlicher Zellen ist bei lichtmikroskopischer Analyse in hohem Maße verändert, wenn lebende mit toten Zellen verglichen werden. Außerdem kann das Aussehen von Pilzzellen sehr stark von der Verwendung verschiedener gebräuchlicher Präpariermedien abhängen. Schließlich erhöhen ontogenetische Veränderungen den Grad der zu beobachtenden Variabilität. Taxonomische Arbeit muß sich jedoch auf Organe in kompatiblen Zuständen und Entwicklungsstadien beziehen. Es wird dringend angeraten, Analysen auf die Morphologie lebender, noch unveränderter Zellen frisch gesammelter Fruchtkörper zu gründen. Lebende Ascocarpzellen müssen im Zustand maximaler Wassersättigung unter Verwendung wässriger Lösungen mit sehr niedriger Konzentration gelöster Stoffe untersucht werden. Leitungs- oder Regenwasser sind hierfür geeignete Präpariermedien. Die Vitaltaxonomie ermöglicht den Gebrauch vieler zusätzlicher und oft neuer Merkmale von taxonomisch hoher Relevanz und oft erhöhter Konstanz. Diese können mit einfachen, zeitsparenden Methoden am lebenden Objekt beobachtet werden; sie sind jedoch im Herbar verwischt oder verschwinden gänzlich. Dies betrifft insbesondere verschiedene Typen von Plasmaeinschlüssen. Das Entwicklungsstadium eines Organs kann im Lebendzustand viel präziser erkannt werden. Die Vitalität von Einzelzellen läßt sich am osmotischen Verhalten, am Aussehen des Zellplasmas oder mithilfe der Vitalfärbung durch basiche Farbstoffe erkennen. Auffällige Veränderungen beim Absterben von Pilzzellen sind z. B. das Schrumpfen um etwa 30-50% vom Ursprungsvolumen, oder die Quellung innerer Zellwandschichten um das ca. 2 bis 5-fache ihrer natürlichen Dicke. Eine neue Kombination wird vorgeschlagen: **Allophylaria nervicola** (Velen.) Baral *comb. nov.*

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1. INTRODUCTION

During my taxonomic studies on Ascomycetes (mainly discomycetes) for some 17 years, I soon noticed that fungal cells mostly show a very different micromorphology under the LM (magnification 600-1500x) when fragments of fruitbodies from the herbarium in tap water mounts were compared with those from freshly collected specimens in the same medium. These alterations were also produced when fresh collections were treated without enough care: during transport to the laboratory by prolonged exposure to a dry atmosphere, by mechanical damage of the fresh ascocarps, or by strong pressure on the coverglass during preparation.

The observed differences between fresh and dried or carelessly treated specimens were, e.g. (FIG. 1 abc→def): (1) dramatic shrinkage of spores, asci and paraphyses; (2) spores clustered at the top of the ascus versus dispersed throughout the (shrunken) ascus; (3) many globose refractive guttules (LBs) within the spores versus several large aggregations or none; (4) refractive bodies within the paraphyses versus "empty" paraphyses.

More detailed investigations using basic dyes for staining the cells *in statu vivo*, as well as osmotic tests, clearly proved that the observed differences originate in the living versus dead state of the cells. Since living fungal cells turned out to offer much more valuable and consistent taxonomic characters (with regard to both cytoplasm *and* cell wall), I early restricted my studies intuitively to the morphology of living cells (except for apical ascus wall structures), a method which I finally called *vital taxonomy* (BARAL, 1989a: 120; 1990). This is in contrast to the currently

applied method of Ascomycete taxonomy using the LM, which is mainly based on describing dead dried material preserved in herbaria.

STRUGGER (1949) gave a comprehensive summary of this problem for the field of plant physiology. He emphasized the study of the living cytoplasm (*Lebendzytologie*) which, he was convinced, would undoubtedly attain more importance in the future when compared to the classical method of studying fixed material (*Fixierungszytologie*). According to STRUGGER (1949: 2, 143), differences in the structure and organization of the living cytoplasm must not necessarily produce differences in the morphology of the cell wall. STRUGGER's *Lebendzytologie*, however, scarcely influenced the current methods of micromorphological research on fungi in the following decades. Instead, the new technique of electron microscopy which *imposes the restriction of viewing only dead or rapidly dying material* (READ et al., 1982: 2062) started its rapid development. More and more taxonomic work is carried out using this technique.

During my study on Leotiales, I became convinced that many taxonomic conflicts are due to the prevailing absence of careful studies of living cells. Only a few mycologists have emphasized the advantage of microscopic examination of Ascomycetes *in statu vivo*: BOUDIER (1885: 95; 1886: 141; 1914: 54) layed stress on the fact that he never used dried specimens for his descriptions because these nearly always give incorrect results. He consequently considered dried specimens *un obstacle à toute bonne classification*. LAGARDE (1906: 135ff.) wrote: *Les échantillons secs d'herbier (...) donnent toujours des résultats médiocres et exposent à des erreurs. Les organes délicats des Discomycètes charnus subissent par la dessication des altérations profondes, irrémédiables.* In her study on the ontogeny of ascospore ornamentation in the Pezizales, LE GAL (1947: 78) wrote: *(...) seules les observations vitales pouvaient nous donner des résultats satisfaisants.* Even anatomical studies of apothecia are, according to CORNER (1929: 264), better performed with living cells: *The best method of examining the growth of the hyphae and the origin of the tissues was found to be by means of freehand sections of living material.*

Surprisingly few reports of method-dependent alterations in fungal micromorphology have been published. Instead, taxonomists tend to describe either living or (more frequently) dead elements without taking possible alterations into consideration and rarely give detailed indications of their preparative treatments by specifying these for each given measurement or illustrated organ. GRADDON (1951: 693) wrote: *The following descriptions are drawn from fresh material and spore measurements are taken from spores freely thrown by living specimens and collected on coverglasses*, but he sometimes described dead elements as well without commenting on this fact. This can often be concluded from his drawings which, in the case of living specimens, show spores clustered at the top of the asci, containing globose and symmetrically arranged guttules. Personal communication revealed that others are used to ignore the living cells as "abnormal" and to prefer the dead elements for study.

Often, however, one is unable to recognize the state of the measured and described elements in a given publication. Recently, SPAIN (1990) reported drastic

alterations induced by reagents applied to fresh spores of Endogonaceae, stating that *it may not be possible to discern from diagnoses whether alterations took place before or after descriptions were made*. He recommended that diagnoses should be given based on both fresh, untreated and reagent-treated specimens. Likewise, HUHTINEN (1990b) wrote: *At least one of the depicted populations should represent living specimens mounted in water*.

My field of work concentrates on the species of the Leotiales. Therefore, most observations of taxonomical importance concerning vital taxonomy were made on this group. A key to the presently known European species is in preparation. This key will be based, as far as possible, using vital taxonomy, and is hoped to allow a rapid identification of fresh collections.

2. MATERIALS AND METHODS

A Zeiss microscope with bright field and phase optics was used for all observations, with phase 100x/1.25 oil immersion objective, and 12.5x or 15x binoculars. The illustrations were made without the aid of a drawing tube. The depicted specimens originate from the following collections (HB = the authors herbarium, A = Austria, CH = Switzerland, D = F. R. Germany, F = France, L = Luxembourg, S = Spain):

Aleuria aurantia (Pers.) Fuck., 25.XI.87, D-Tübingen-Pfrondorf, on clayey ground, leg. HB, HB 3316 -- **Allophylaria nervicola (Velen.) Baral comb.nov.** [2], 24.X.87, D-Tübingen-Pfrondorf, on petioles and veins of **Acer pseudoplatanus**, leg. HB, HB 3292 -- **Brunnipila clandestina** (Bull. ex Mérat : Fr.) Baral in Baral & Kriegl-st., 30.V.88, D-Tübingen-Pfrondorf, on canes of **Rubus idaeus**, leg. HB, HB 3425 -- **Ciboria caucus** (Rebent.) Fuck., 16.III.88, D-Kirchheim-Bonlanden, Breuningsweiler, on male catkins of **Alnus glutinosa**, leg. J. Haedecke -- **Cistella deflexa** (Gradd.) Raitv., 3.XI.85 D-Tübingen-Lustnau, on leaves of **Populus** ? **canadensis**, leg. HB, HB 2951 -- **Conchatium fraxinophilum** Svrček, 24.X.87, D-Tübingen-Pfrondorf, on petioles of **Fraxinus excelsior**, leg. HB, HB 3293a -- **Discina ancilis** (Pers.) Sacc., 26.III.88, D-Tübingen-Pfrondorf, on bark of **Picea abies** trunk, leg. HB -- **Hymenoscyphus** cf. **sazavae** (Vel.) Svrček, ≈27.IX.87, F-Gérardmer, on wood and cone of **Picea abies**, leg. J. Deny, HB 3277 -- **Hymenoscyphus scutula** (Pers. : Fr.) Phill., 24.X.87, D-Tübingen-Pfrondorf, on stems of **Tanacetum vulgare**, leg. HB, HB 3290 -- **Hymenoscyphus consobrinus** (Boud.) Hengstm., 24.VIII.87, Tübingen-Dettenhausen, on stems of **Anthemis nobilis**, leg. G. Haupter -- **Lachnum controversum** (Cke.) Rehm, 24.VII.87, D-Tübingen, on culms and leaves of **Phragmites communis**, leg. HB, HB 3229 -- **Lasiobelonium corticale** (Pers. : Fr.) Raitv., 15.V.88, L-Tuntange-Hollenfels, on bark of **Populus tremula** stump,

[2]) Basionym: **Helotium nervicolum** Velenovský, Monogr. Discom. Bohemiae 1934: 206 (≡ **Conchatium nervicolum** (Velen.) Svrček, = **Allophylaria subhyalina** forma b in BARAL & KRIEGLSTEINER 1985: 94)

leg. C. Besch, R. Swart-Velthuyzen & HB, HB 3386 -- **Lasiobelonium variegatum** (Fuck.) Sacc., 28.VII.88, CH-Schaffhausen-Thayngen, on bark of **Salix** = **cinerea** branch, leg. HB & G. Marson, HB 3496 -- **Lecanora conizaeoides** Nyl. ex Crombie, 13.VII.88, D-Tübingen-Pfrondorf, on wood of ? **Picea abies** fence, leg. HB, HB 3459 -- **Melastiza chateri** (W.G. Smith) Boud., 20.XI.87, CH-Schaffhausen-Thayngen, on clayey ground, leg. HB & P. Blank, HB 3317 -- **Mniaecia jungermanniae** (Nees ex Fr.) Boud., 12.II.88, D-Tübingen-Pfrondorf, on **Cephalozia bicuspidata**, leg. HB, HB 3336b -- **Mollisia** spec., 20.VI.88, D-Tübingen-Pfrondorf, on wood of **Quercus** trunk, leg. HB, HB 3441 -- **Nimbomollisia eriophori** (Kirchn.) Nannf., 21.V.88, CH-Zug-Unterägeri, on culms of **Juncus effusus**, leg. J. & L. Rothenbühler -- **Nimbomollisia melatephroides** (Rehm) Nannf., 27.VII.88, CH-Zug-Unterägeri, on leaves & culms of **Molinia coerulea**, leg. HB, P. Blank, J. & L. Rothenbühler, HB 3483 -- **Orbilia auricolor** Blox. ex Berk., 21.VIII.88, D-Tübingen-Pfrondorf, on stems of **Oenothera biennis**, leg. HB, HB 3527 -- **Orbilia delicatula** (Karst.) Karst., 22.VIII.88, D-Tübingen-Pfrondorf, on wood of **Acer pseudoplatanus** stump, leg. HB, HB 3529 -- **Orbilia** ? **rosella** (Rehm) Sacc., 10.VIII.88, D-Tübingen-Pfrondorf, on stem of **Melilotus albus**, leg. HB, HB 3518a -- **Orbilia sarraziniana** Boud., 14.V.88, L-Beaufort, on wood of **Fagus sylvatica** twig, leg. HB -- **Orbilia** spec., 10.VIII.88, D-Tübingen-Pfrondorf, on stem of **Melilotus albus**, leg. HB, HB 3518b -- **Pezicula cinnamomea** (DC. : Pers.) Sacc., 23.VII.87, D-Tübingen-Pfrondorf, on bark of **Carpinus betulus** twig, leg. HB, HB 3239 -- **Pezicula livida** (Berk. & Br.) Rehm, 27.VIII.86 D-Tübingen-Pfrondorf, on bark of **Pinus sylvestris** branch, leg. HB -- **Peziza** ? **fimeti** (Fuck.) Seaver, ≈30.V.88, D-Tübingen-Pfrondorf, on straw and horse dung, leg. P. Zinth & HB -- **Phaeohelotium geogenum** (Cke.) Svrček & Matheis, 18.X.89 A-Grünburg-Steinbach, on leaf & twigs of ? **Quercus**, and apple pressings residue, leg. H. Helm, HB 3907 -- **Polydesmia pruinosa** (Jerdon in Berk. & Br.) Boud., 14.VI.87, D-Tübingen-Pfrondorf, on **Hypoxylon** spec., leg. HB -- **Pyrenopeziza petiolaris** (Alb. & Schw. : Fr.) Nannf., 26.III.88, D-Tübingen-Pfrondorf, on petioles of **Acer pseudoplatanus**, leg. HB -- **Rutstroemia elatina** (Alb. & Schw. : Fr.) Rehm, 15.III.1986 D-Tübingen-Pfrondorf, on bark of **Abies alba** twigs, leg. HB -- **Sarcoscypha austriaca** (Beck ex Sacc.) Boud., 20.V.79, D-Hinterzarten, Feldberg, on wood of **Acer pseudoplatanus** branches, leg. HB, P. & D. Laber, HB 2537 (neotype) -- **S. coccinea** (Scop. : Fr.) Lamb., 12.III.79, D-Karlsruhe-Hambrücken, on wood of **Ulmus carpinifolia** branches, leg. K.H. Waßmuth & HB, HB 2460 (neotype) -- **S. jurana** (Boud.) Baral, 5.III.79, D-Hayingen-Lauterach, on bark of **Tilia platyphyllos** branches, leg. P. Zinth & HB, HB 2461 -- **S. macaronesica** Baral & Korf in Baral, 20.I.82, S-Gomera-Vallehermoso, on wood of ? Lauraceae twigs, leg. P. Zinth, HB 2610 (holotype) -- **Scutellinia scutellata** (L. ex St. Amans) Lamb., (collection of unknown provenance), leg. HB -- **Trichopezizella nidulus** (Fr.) Raitv. s.l., 11.V.88, F-Gérardmer, on stems of **Ranunculus aconitifolius**, leg. J. Deny, HB 3384 -- **Tubeufia cerea** (Berk. & Curt.) Booth, 17.VI.86 D-Murrhardt, on **Diatrype stigma**, leg. L. Krieglsteiner, HB 3039 -- **Tubeufia paludosa** (Crouan & H. Crouan) Rossm., 7.VIII.88, D-Tübingen, on culm of **Phragmites communis**, leg. HB, HB 3507b -- **Tympanis alnea** (Pers.) Fr., 25.V.87, CH-Schaffhausen-Thayngen, on bark of **Prunus avium** twig, leg. P. Blank, HB 3187 -- **Verpa digitaliformis** Pers., 8.V.91 D-Tübingen-Bebenhausen, under **Petasites**, leg. HB, HB 4411 -- **Xanthoria parietina** (L.) Th.

Fr., 7.VI.86 D-Tübingen-Pfrondorf, on bark of **Malus domestica** trunk, leg. HB, HB 3036.

Abbreviations:

✻ = living hydrated state *(in statu vivo et udo/umido)*

† = dead hydrated state *(in statu emortuo et udo/umido)*

LM = light microscope
TEM / SEM = transmission / scanning electron microscope

H_2O = tap water (of medium hardness)
IKI = Lugol's solution (1%[3] I_2, 3% KI in water)
MLZ = Melzer's reagent (1.2% I_2, 3.6% KI, 48% chloral hydrate in water)
CRB = Brilliant cresyl blue (0.1-1% in tap water, not distilled water)
CRB_A = alkalinized CRB: a small drop of 0.5% KOH or strong NH_4OH is added to a CRB mount
KOH = potassium hydroxide (0.5-10% in water)
CB = cotton blue (0.5% in lactophenol, i.e. equal proportions of phenol, glycerol, lactic acid and water)
CZB = chlorazol black (1% in glycerol buffer)

cytoplasmic structures:
LB = lipid body
SCB = KOH-soluble cytoplasmic body
WB = Woronin body
N/NO = nucleus/nucleolus

vacuolar structures:
VB = refractive vacuolar body
V = non-refractive vacuole
MC = metachromatic corpuscle

DBB = de Bary bubble

3. HOW TO RECOGNIZE LIVING FUNGAL CELLS

Vitality of single cells is defined according to STRUGGER (1949: 170f.) by intact (semipermeable) plasma membranes. Viable cells therefore show turgescence when mounted in hypo-osmotic media (with an osmosity lower than that of the cell sap). Current tests, such as for germination ability or metabolic activity, are here of minor value because (1) vitality has to be proved for a single cell, and (2) many types of cells (e.g. asci) are unable to germinate.

[3]) All values in this paper given in % refer to w/w = weight of solute per weight of solution (solvent + solute).

Vitality can easily be recognized under the LM (oil immersion) by the following tests: 1. Cell turgor: adding strongly hyperosmotic media to the water mount provokes shrinkage (not collapse!) of living but not of dead cells (in the case of elastic cell walls). Reversible plasmolysis is also a reliable proof for vitality, occurring especially in vegetative cells. These tests require that the cell wall is freely permeable for the reagent, otherwise both living and dead cells may collapse. In water mounts of multi-celled organs, the septum is strongly curved towards the dead cell if the adjacent cell is intact (FIGs. 2d, 3). Slight constrictions at the septa are typical for living thin-walled cells (FIGs. 2 a-c, 7a, 13a).

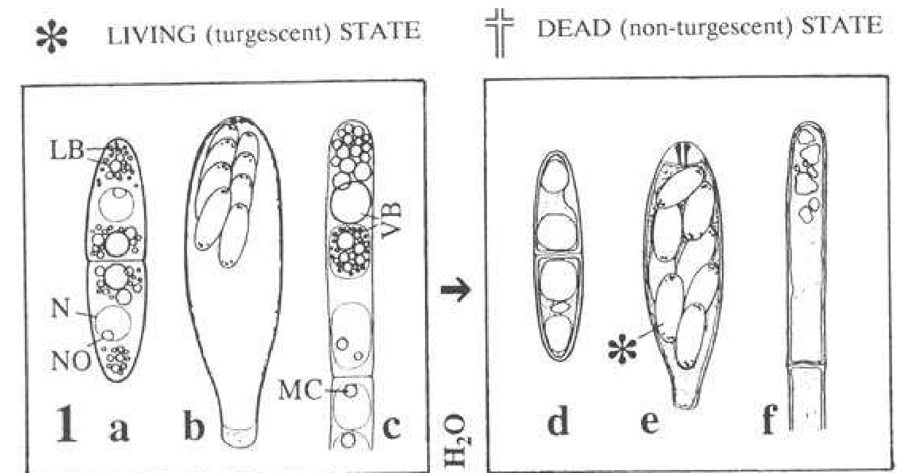


FIG. 1. How to distinguish living from dead cells. Hymenial elements (spore, ascus, paraphysis) of fictitious species of Leotiales in the living (**abc**) versus dead state (**def**). The dead ascus contains still living spores.

2. Structure of the cytoplasm: in water mounts, living fungal tissue shows high transparency and contrast while dead cytoplasm is detached from the cell wall and is usually much more refractive due to dehydration, therefore opaque (FIGs. 1 d,f; 2 d,f; 3; 12b). Cytoplasmic inclusions show aesthetic and symmetrical patterns when intact but irregular distortion or optical absence when dead.

3. Staining *in statu vivo*: basic (alkaline) dyes added to water mounts accumulate (within seconds or minutes) selectively inside intact vacuoles of the living cell without damage to the plasma membranes, either homogeneous (FIG. 30, *VB*) or by forming globose bodies as a result of flocculation of (poly-)phosphates (volu-

tin bodies, metachromatic corpuscles/granules, FIGs. 4, 30, *MC*). The living cytoplasm is thereby not stained. Yet, in dead cells with destroyed plasma membranes (loss of semipermeability), the cytoplasm is deeply stained within seconds while no accumulation and MC-formation occurs in the vacuoles (FIG. 21b; GUILLERMOND, 1941: 129ff.; STRUGGER, 1949: 126ff.; HEINEMANN, 1956: 36ff.; BANCHER & HÖFLER, 1959: 150ff.; HOHL, 1987: 17; ROMEIS, 1989: 27, 302ff., *Trypanblau-Ausschlusstest*). Staining of the vacuoles with basic dyes is *essentially a phenomenon of the living cell* (GUILLERMOND, 1941: 145) and the most valuable test for vitality (BANCHER & HÖFLER, 1959: 153).[4]

The contrast between the unstained (living) and stained (dead) cytoplasm is usually striking and corresponds to fig. 119 in ERB & MATHEIS (1983) showing both living and dead cells in multi-celled spores of **Ophiobolus acuminatus** stained by phloxine. (Similar to basic dyes, phloxine is only able to stain the dead cytoplasm.) Yet, cell contents rich in lipids or vacuoles but poor in cytoplasm are less distinctly stained in the dead state, and thick walls of spores were found to be impermeable to basic dyes in either state.

[4]) Overstaining must be avoided since concentrations above approx. 0.5% may be lethal. Aqueous CRB solution, slightly alkalinized (CRB_A) in case the stain does not readily penetrate through the plasma membranes, gives good results: blue-violet for MCs, blue-green to violet for homogeneous staining (see CHADEFAUD, 1938: 116; GUILLERMOND, 1941: 143; LE GAL, 1947: 78). The CRB (0.1-1%) is added to the margin of the water mount resulting in a CRB solution of about 0.05-0.5%. The KOH accelerates the process of staining the vacuoles because only undissociated molecules of CRB (which do not exist at a pH lower than 7) pass through the plasma membranes.

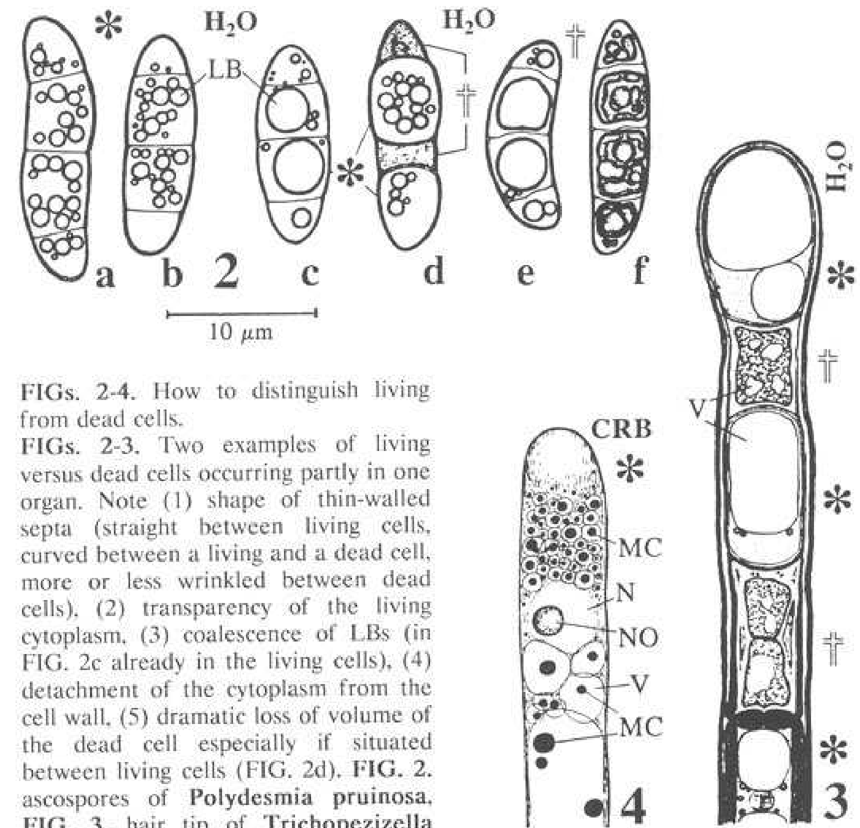
Non-alkalinized CRB is also used for diagnostic violet stains of wall layers in Basidiomycetes (SINGER, 1986: 89). I recommend CRB as a standard reagent for Ascomycete taxonomy because it allows the recognition of mucilage on hyphae or spores by staining deep violet (see chapter 5.c.) while resinous exudates stain deep turquoise-blue.

I found the aqueous solution (in tap water) to be stable for years; yet, CLÉMENÇON (1972) added several ingredients because he found CRB in water to precipitate within a few days. CRB is not considered carcinogenic by ERB & MATHEIS (1983: 28). Other basic dyes dissolved in tap water have been tested in comparison: toluidine blue, used by MOORE (1965: 26) and MATHEIS (1975: 160) as a stain for mucilage, gives results comparable to CRB showing striking turquoise-blue and red-violet metachromatic colors. However, CRB gave more reliable results because it is blue in tap water while toluidine blue is violet in that medium. Contrary to toluidine blue, the color of CRB depends furthermore on the pH (turquoise-blue in acetic acid, red-violet in KOH). Contrary to CRB, toluidine blue did not easily penetrate into the living cell. Neutral red and cotton blue may also be used but do not show striking metachromatic color changes.

4. HOW TO AVOID MORPHOLOGICAL ALTERATIONS OF LIVING FUNGAL CELLS DURING MICROSCOPIC EXAMINATION

4.a. Mounting medium

STRUGGER (1949: 4) recommended mounting cells of living land plants in *aqua bidest*. Similar to plants, Ascomycetes, developing under natural conditions, are usually exposed to rain water (i.e. approximately distilled water). Drought-



FIGs. 2-4. How to distinguish living from dead cells.
FIGs. 2-3. Two examples of living versus dead cells occurring partly in one organ. Note (1) shape of thin-walled septa (straight between living cells, curved between a living and a dead cell, more or less wrinkled between dead cells), (2) transparency of the living cytoplasm, (3) coalescence of LBs (in FIG. 2c already in the living cells), (4) detachment of the cytoplasm from the cell wall, (5) dramatic loss of volume of the dead cell especially if situated between living cells (FIG. 2d). **FIG. 2.** ascospores of **Polydesmia pruinosa**, **FIG. 3.** hair tip of **Trichopezizella nidulus**. 2000x.
FIG. 4. Upper region of a living immature ascus with fusion nucleus and vacuoles containing MCs which are typical for living cells (stained with neutral red or CRB; from CHADEFAUD, 1938: fig. 2).

tolerant species in particular, which dry out completely during dry weather, rapidly absorb water during rainfall and are most favourably collected immediately afterwards. Aquatic species develop below the water level in rivers and lakes. Rain or tap water is therefore the natural preparation medium for these fungi.

Mounting in media with a high osmosity induces severe shrinkage, especially of asci (see chapter 5.a.). A test with artificial sea water (3.4% sodium chloride [NaCl]) on some species of Leotiales and Lecanorales revealed shrinkage in ascus width for 2-12%. Accordingly, concentrations upto 0.3% NaCl produce shrinkage for max. about 1% and are therefore compatible to distilled water for the purpose of taxonomy. Tap water has an osmosity of roughly 0.0003 to 0.006% NaCl. Hence, it is safe to say that mounting fruit-bodies of Ascomycetes in tap water of any hardness corresponds to the natural situation of these fungi.

Staining *in statu vivo* with basic dyes or with IKI is only lethal after a considerable period of time. However, mounting in currently used media, with either very high (alkaline) or low (acid) pH or other lethal properties, such as MLZ, CB, glycerol buffer (= *L4*), Hoyer's fluid, lactic acid, 2-10% KOH, kills most types of fungal cells within seconds (LINDER, 1929; BARER et al., 1953: 720; HUHTINEN, 1985: 18; BARAL, 1987a: 409 and unpublished data). CLÉMENÇON (1972: 49) worked only with lethal mountants and did not even mention water as a possible medium. The lethal effect of these mountants should be clear but are actually not well-known. Workers are indeed surprised when accidentally encountering it: e.g., LUARD (1983: 529) noted that CB had an *unexpected effect on the appearance of* ***Chrysosporium fastidiosum*** causing a *dramatic contraction of the cytoplasm*.

These mountants have been introduced for the study of herbarium specimens in order to obtain transparency of the cytoplasm, to dissolve trapped air on imperfectly wetted hyphae, to inflate collapsed cells, to avoid movement of floating spores, and to avoid desiccation of the mount during microscopic study (see FLEMING & SMITH, 1944: 17; BARAL, 1987: 408f.). In order to get compatible results, these mountants are often also used for fresh specimens. This led CLÉMENÇON (1972, *L4*) to employ lower concentrations (20% glycerol, ≡ 8.5% NaCl[5]) in order to avoid the collapse of living cells. In my experience, mature spores with rigid walls, e.g. in Pezizales and Xylariales, often survive some hours in media such as MLZ, thus killing by heating of the slides is necessary in order to obtain results compatible with the dead specimen.

Some mycologists try to avoid inflation of fungal cells to an "unnatural" oversize by employing "isotonic media", e.g. 1% glucose (≡ 0.17% NaCl) or 0.85% NaCl (CHADEFAUD, 1938: 116; BRUMMELEN, 1967: 18; ERB & MATHEIS, 1983: 13, 15). The glucose medium is, however, strongly hypo-osmotic and thus gives results corresponding to tap water mounts. H. CLÉMENÇON (in litt. 20.7.87) believed that

[5]) The given concentration of NaCl solution has the same osmotic pressure (osmosity) as the mentioned solution. An osmosity of 1 g-mol/l = 5.6% NaCl is equivalent to 6 x 10^{23} ions per litre

the physiological state of fungal cells is usually characterized by a low turgor pressure clearly distinct from the state of full hydration and maximum tugor which is obtained in mounts of distilled or tap water. Since the osmotic pressure of fungal cells strongly varies among species and even organs, each case would then need its special iso-osmotic mountant. The following test proves, however, that maximum turgor and hydration is the natural state for full metabolic activity in ascocarp cells of discomycetes: I compared mounts of fresh ascocarps of several species in tap water with those made instead in oil, e.g. immersion oil (or paraffin oil according to STRUGGER, 1949: 5). The oil prevents a possible uptake of further external water during preparation. No difference in size and appearance of the cells and their contents could be observed. This should hold true for all fungal structures which are naturally exposed to rain water. Cells having a cell wall do not need an iso-osmotic medium: their physiological medium is, in these cases, close to distilled water, i.e. strongly hypo-osmotic.

I therefore use water as a standard medium for obtaining compatible measurements of living fungal cells. Likewise, MAIRE (1926: 44), for example, wrote: *Les dimensions des spores doivent être mesurées autant que possible sur des spores fraiches examinées dans l'eau*, and SPAIN (1990: 71) wrote: *Arguments are presented for diagnoses based on the morphology of fresh spores suspended in water*.

Osmotolerant hyphae, able to grow in media with a high concentration of glucose, and especially marine fungi might represent an exception. LUARD (1983: 533) therefore measured the hyphal diameter of osmotolerant species by mounting in *slightly hyperosmotic KCl*. A similar case is represented by the ascospores while still inside the living ascus: the spores are embedded in the vacuole sap with a high osmosity, and increase considerably in size within about 1/2 minute following discharge into water.

In order to obtain compatible measurements, I recommend to avoid measuring ascospores within living asci. Probably all fungi can be observed and measured in the standard mountant tap water without bursting of the hyphae. This view is supported by the report of JONES et al. (1991) who found fungal cultures to survive several years in distilled water in small phials.

4.b. Preparative techniques in vital taxonomy

The specimens chosen for microscopic examination must be in the fully hydrated living state. Species growing in permanently humid places are usually sensible to drought and must be transported to the laboratory without the loss of intracellular water. Desiccated ascocarps of drought-tolerant species can be rehydrated by spraying with water several minutes prior to preparation. Fragments of the fruit-bodies are gently (!) squeezed under the coverglass. Freehand sections (about 30-100 μm thick, depending upon the diameter of the cells) through the fully hydrated living fruit-body made with a razor blade are superior, allowing excellent study of ascocarp textures (BOUDIER, 1886: 136; CORNER, l.c.), while dead hydra-

ted tissue is difficult to cut freehand because of its flabbiness. Heating or any stronger pressure must be avoided. Very often a certain number of spores, asci, and vegetative cells are already dead prior to preparation (FIGs. 2d, 3), or they die when cut by the razor blade. These cells must be disregarded for the purpose of vital taxonomy.

5. ALTERATIONS IN THE CELL WALL

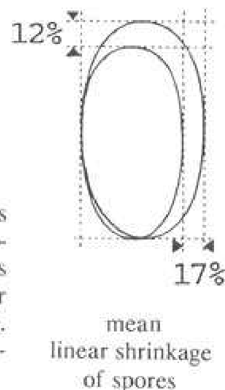
5.a. Cell size: the shrinking effect

As hydrated fungal cells die, they lose turgor and often show a strong decrease in size (without collapsing) due to elasticity of the cell wall and loss of water mainly from the vacuoles. Irreversible shrinkage is induced by lethal substances such as, e.g., 50% chloral hydrate, 1-5% KOH, or simply by mechanical pressure (FIGs. 2, 3, 6, 8, 9, 10, 12, 13, 14, 21, 22, 33, 34, 35), which destroy the semipermeable properties of the plasma membranes. Both length and width are not infrequently reduced for about 10-20%, with the cell volume (including the cell wall) for about 30-50% (TAB. 1). Such variation in measurements is very often employed for differentiation among species! A linear shrinkage in width for about 30-57% was observed in the asci of species of **Lecanora** (FIG. 9), **Xylaria** and **Lasiosphaeria**.

HUHTINEN (1985: 18) wrote: *In numerous taxa of both the Helotiales and Pezizales, I have observed that the mountants commonly in use have a shrinking effect. When dried material is revived with Melzer's reagent or lactic acid, the sections do not always regain their original dimensions. This can be concluded from the results of adding these mountants to natural or water mounts of fresh apothecia. Shrinkage of 5-15% takes place immediately, due to a loss of turgor in the cells.*

decrease in %	ASCI length	ASCI width	ASCI volume	SPORES length	SPORES width	SPORES volume
Mn. jung.	18.5-20.3	17.8-21.7	46-51	5.7- 8.3	13.9-21.5	30-44
Ci. caucus	14.9-19.7	18.7-22.9	44-51	12.1-14.0	20.8-24.6	45-51
Ru. elat.	10.6-17.4	17.2-23.8	40-52	12.1-18.2	13.3-16.7	34-42
Pe. amenti	13.1-20.8	15.1-25.6	37-56	9.8-13.9	7.5- 9.8	24-29

TAB. 1. Irreversible shrinkage in Leotiales (tap water versus MLZ). **Mniaecia jungermanniae**, **Ciboria caucus**, **Rutstroemia elatina**, **Pezizella amenti**. 4 asci and 4 spores from each species were measured in the living state and after MLZ was added by direct visual monitoring of shrinkage. (Care must be taken in dead spores not to measure the plasma body only).



mean linear shrinkage of spores

BECKETT et al. (1984: 93) reported severe dimensional changes during preparative procedures for the SEM: *Although it is logical to expect biological specimens which normally have high water contents to shrink when dried (...), few published results adequately account for this.*

The following literature also discusses the shrinking effect for fungal cells: DE BARY (1887: fig. 43, see FIG. 6), LAGARDE (1906: 135), MAIRE (1926: 44f.), STEINER (1957: 249), HERTEL (1967: 3, 13% linear shrinkage in KOH vs. H_2O), DRING (1971 [in LUARD 1983: 529]), HEIN (1976: 16, about 15% linear shrinkage in CB vs. H_2O), DÖBBELER (1984: 206), BARAL (1987a: 409; 1987b: 121; 1989a: 120; 1989b: 222), HUHTINEN (1990a: 64, 68, shrinkage in width ca. 15%, CB or MLZ vs. *in statu vivo*, H_2O).

The percentage of linear shrinkage during the death of the cells strongly depends upon the taxon, cell type, and cell axis (TAB. 1), but also upon the mountant in which the dead cells are measured (HUHTINEN, l.c.). Furthermore, rehydrating dead cells may result in damaged profiles: e.g., ascus width shows higher variation due to (1) strong pressure on the coverglass which may flatten dead asci, and (2) irregularly arranged spores which swell out the ascus wall (FIG. 9b). Hairs of **Brunnipila** (Hyaloscyphaceae) collapse in dehydrating reagents: their apparent width depends on the angle at which they are lying and can therefore vary from about 1 to 7 µm. The width of the hydrated living hair is about 3-5 µm.

KOH is commonly considered an agent which increases the volume of fungal cells, especially of spores (e.g. HEINEMANN & RAMELOO, 1985), or restores dried plant cells to their "original size" (e.g. CUNNINGHAM, 1969). Such a swelling effect is mainly observed when KOH mounts are compared with mounts of dead cells in water, CB, MLZ etc. When applied to water mounts of living fungal cells, however, KOH often provokes dramatic shrinkage, especially with asci.

On the other hand, living mature spores which pass into the germination phase show a considerable increase in size in many species, partly due to synthesis of new cellular wall material (GARRAWAY & EVANS, 1984: 221). Therefore, even in the dead, shrunken state with a turgor nearly zero this increase in size is obvious. In other species the spores do not change their dimensions during germination. Consequently, measurements which do not indicate (1) the state (living or dead) of the measured cell, (2) the mounting medium, (3) the preparative treatment (heating, mechanical pressure), and (4) the development stage of the cell, are of minor taxonomic value (see also HUHTINEN, 1990a; b).

Decrease of the cell volume with the decrease of the hydrostatic pressure (turgor pressure) is a well-known phenomenon in plant and animal cells and tissue (DAINTY, 1976). The elastic modulus of the cell wall controls the rate of swelling or shrinking: cells with rigid walls do not shrink noticeably if the maximum turgor pressure is brought down to about zero; their elastic modulus is high. Highly elastic cell walls, however, have a very low elastic modulus and the cells shrink considerably in this case. Furthermore, cells shrink to a higher extent if the maximum turgor pressure is rather high, e.g. in mature asci.

It appears therefore incredible that, e.g., CUNNINGHAM (1972) wrote the following incorrect statements about a mountant containing 67% chloral hydrate: *AH*[6] *does not distort spore measurements*, and: *AH sometimes causes slight temporary plasmolysis of certain fresh fungi but normal turgor is totally restored almost always within a few minutes*! CUNNINGHAM misapplied the term *turgor* to cells in a dead, non-collapsed state, their walls showing an even, non-shriveled outline but a very low tension. Likewise, LINDER (1929) and FLEMING & SMITH (1944: 17) wrote that lactophenol *immediately restores the turgor* and *rarely causes either swelling or shrinkage*.

Shrinkage of asci immediately after spore discharge is a well-known phenomenon (FIGs. 23-25; BULLER, 1931: 247: *an ascus reduces its volume to about one half on exploding*; INGOLD, 1986: fig. 1). However, shrinkage to a comparable extent during the death of the mature ascus before *the spores are released* (FIG. 8 a→b, 9 a→b, 10 a→b) was very rarely reported (e.g. by DE BARY, see FIG. 6) though generally occurring. Shrinkage of asci without spore liberation may also be obtained reversibly without killing by using, e.g., 1 M (= 30%) saccharose (≡ 4.4% NaCl) (INGOLD, 1953: 17, fig. 8). With saccharose solutions higher than about 45% (≡ 9% NaCl) I was able to shrink the asci of **Lecanora conizaeoides** in width from 20-21.5 μm (in tap water) to 12-13 μm (i.e., to an extent close to FIG. 9b), and to reverse these asci to their original size and shape by replacing the saccharose solution with water.

Unawareness of shrinkage has resulted in numerous conflicts and misinterpretations. E.g., KORF (1951) found roughly 25% lower measurements of hairs, asci, spores, and paraphyses in some 20 years old type material of two species compared with the original description given by GRELET. Since GRELET usually studied living cells (which can be concluded from his drawings), following the tradition of BOUDIER, the discrepancy discovered by KORF is readily explained by the shrinking effect. KORF, however, concluded that GRELET's measurements were incorrect, *presumably because of an error in the microscope calibration*, and therefore multiplied GRELET's measurements by an assumed error factor of 0.75. Likewise, authors have found BOUDIER's measurements (which he gave in the text) to be usually too high when compared with their own measurements. MAIRE (1926: 47) referred this discrepancy to an error in the construction of BOUDIER's measuring scale, and recommended the subtraction of one-tenth from BOUDIER's values. Clearly, the shrinking effect is at least one of the true reasons for BOUDIER's larger measurements. Indeed, the magnification of *820x* indicated by BOUDIER on his plates *after 1885* is considered by v. BRUMMELEN (1969; and also by me) to be correct while plates *before 1885* are *approx. 840x* instead of "340x" as BOUDIER indicated (BRUMMELEN, l.c.). The values given in BOUDIER's texts after 1885 are, however, highly erroneous in some cases.

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[6]) AH = Andre's modification of Hoyer's fluid: arabic gum 15g, chloral hydrate 100g, glycerol 10g, water 25ml

Some striking effects:

1. Spore arrangement: spores conspicuously change their arrangement during the death of the mature ascus due to excessive loss of water from the large vacuole of the ascus. This is of taxonomic importance since the arrangement of spores in the asci is very often mentioned in species descriptions. Three phenomena may be observed:

(1) The spores move towards the base of the ascus, thus the *pars sporifera* increases considerably in length (FIG. 8, **Mniaecia**, from 67 to 135 μm; FIG. 9, **Lecanora**, from 26.5 to 38.5 μm). Strictly biseriate spores thereby often become totally (or only in the lower part) uniseriate (FIG. 8).

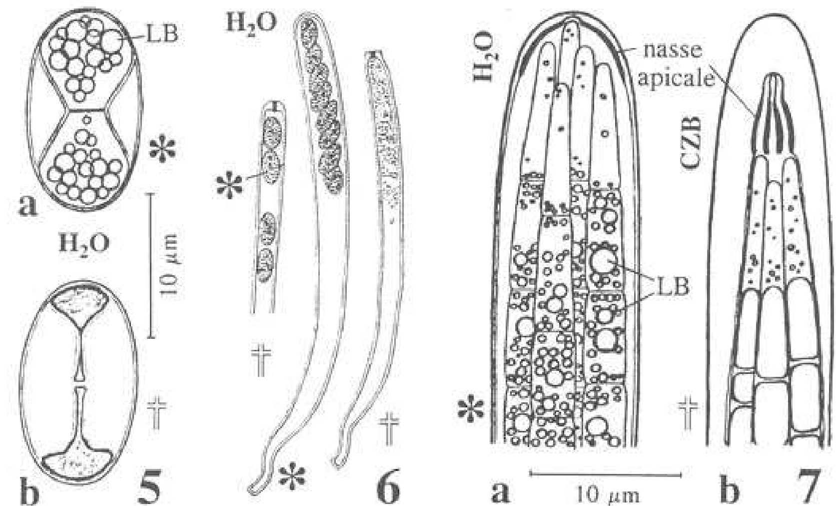
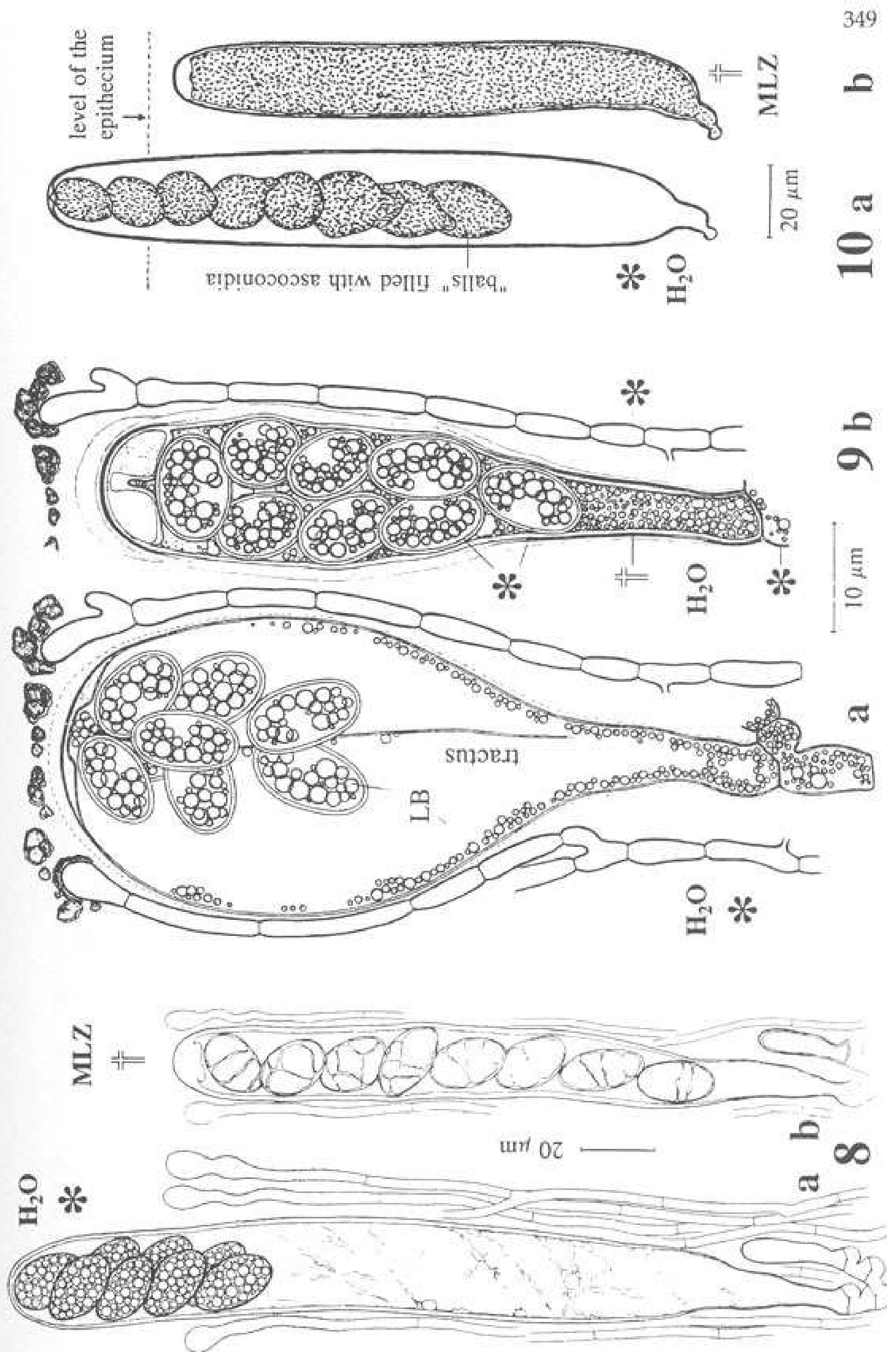


FIG. 5. Expanding wall phenomenon in the ascospores of **Xanthoria parietina**. Note coalescence of single LBs. 2000x.
FIGs. 6-10. Micromorphological alterations of asci (FIGs. 7-10 show one ascus before and few minutes after killing). Note (1) shrinkage of asci and retraction below the tips of paraphyses, (2) loss of most of the vacuole sap, (3) expansion of apical wall layers, (4) movement of spores towards the base of asci. FIG. 9a might be the first published figure of a living Lecanorales ascus! **FIG. 6. Sclerotinia sclerotiorum** (from DE BARY, 1887: fig. 43), **FIG. 7. Tubeufia cerea**, (2000x), **FIG. 8. Mniaecia jungermanniae** (500x), **FIG. 9. Lecanora conizaeoides** (1500x), **FIG. 10. Tympanis alnea** (500x).



(2) Filiform spores lying as straight bundles parallel to the long axis of the ascus may become spirally twisted (e.g. in **Vibrissea**).

(3) Ascoconidia packed inside 4-8 "balls" become disarranged and continuously fill the whole ascus (FIG. 10).

2. Living spores within mature living asci mounted in water are narrower when compared with discharged spores due to the ascus turgor (high osmosity of the large vacuole): in some tested species of Leotiales and Rhytismatales, they were 9-15(-30)% narrower within the asci while their length was nearly unchanged.

3. Protuberant asci: asci shrink much more in length compared to paraphyses. In the living state, mature asci often greatly exceed paraphyses in length but retract below the level of the latter on killing or after spore discharge (FIGs. 8, 10, 33; DE BARY, 1887: 87, 92; BULLER, 1931: 247). Statements such as "asci covered by an epithecium" (composed of agglutinated apical cells of the paraphyses) often originate from dead material. Due to their turgor, living Lecanorales asci tear crevices in the epithecium (long before spore discharge takes place) by pushing the paraphyses aside (FIG. 9).

4. Septate thin-walled hyphae or spores show slight constrictions at the septa *in statu vivo* due to the internal turgor, but an even surface *in statu emortuo* (FIGs. 2 ab→ef, 13 a→b, 34 a→b). The septum is often irregularly wrinkled in the dead state (FIG. 2 ef).

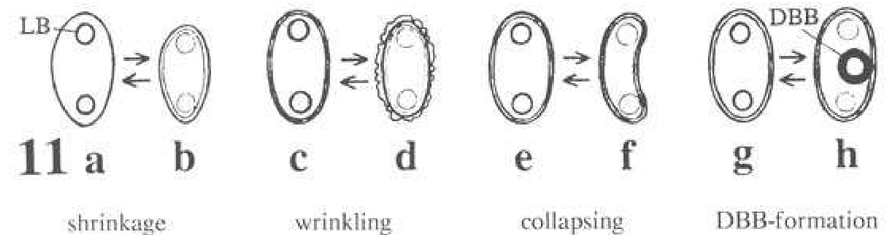


FIG. 11. Four reactions of a living cell on water loss, induced either by desiccation, or by a medium with a high osmosity.

5. False ornamentation: Ascospore walls of Pezizales are often composed of two separable layers, the outer being non-elastic, the inner elastic. As the spores die, the wall loses tension: the inner layer contracts while the outer layer becomes wrinkled by separating more or less from the inner layer. This is, e.g., the case in all studied species of **Sarcoscypha** (BARAL, 1984: fig. 8). Such "false ornamentations" are not stained by CB. ZHUANG (1991) described a new species, **S. striati-**

spora, differing from **S. occidentalis** merely by transverse striations on the spore wall and lower dimensions of spores and asci. Such "characters" are of no use for the delimitation against **S. occidentalis**. LE GAL (1947: 223-238) described a special type of spore ornamentation as *non calloso-pectique*, mainly in members of the Sarcoscyphinae, of longitudinal or transverse striations which are probably also a result of shrinkage of originally smooth spores. READ & BECKETT (1983) reported a reticulate surface texture characteristic of critical point-dried urediospores but a smooth surface in frozen-hydrated material. The authors consider the reticulation to be artificial resulting from shrinkage or the removal of the unfreezable (bound) water from the cell wall.

5.b. Wall thickness: the expanding wall phenomenon (imbibition effect)

Strongly thickened inner wall layers of asci (known as *apical dome/apparatus*, or *tholus*, and approximately corresponding to the endotunica = *Couche D* in REYNOLDS 1989) proved to be very useful in delimiting natural higher taxa. Such thick ascus walls differ fundamentally from solid, constantly thick walls: a thick ascus wall is indicative of the dead state while living asci show dramatically thinner walls (FIGs. 6-10), even in immature asci with a relatively low turgor (DE BARY, 1887: 87, 95; KERR, 1961: 474ff.; BARAL, 1987a: 413; 1987b: 122). Likewise, CHADEFAUD (1944: 9) figured thin and very thick apical apparati in asci of **Leotia**, and correctly referred the strong wall imbibition to the loss of turgor of the ascus vacuoles.

These wall layers represent a swellable, hydrophilous matrix with a considerable but limited expansibility: they become imbibed with water within a few seconds as soon as the asci die, either in water (naturally or by mechanical pressure, FIGs. 6, 9), or by adding lethal reagents (FIGs. 7, 8, 10; see also HOGGAN 1927: 42, pl. V, 6). Increase of about 2-5 times the original thickness usually occurs. The inner contour strongly loses contrast and may severely change its shape (FIG. 9 a→b). KOH is not necessary for the effect as was suggested by HONEGGER (1982: 213, 215) but often produces further expansion when applied to dead asci.

Amyloid layers (except for that part of the amyloid ring which protrudes into the ascoplasm) show this phenomenon also (FIG. 43 b→c). The intensity of the iodine reaction thereby logically decreases with expansion, thus a strong reaction *in statu vivo* changes to a weak or moderate reaction *in statu emortuo*.

TEM-investigations on thickened ascus walls show a loosely fibrillar organization which represents the solid part of the endotunica. The ample space between the microfibrils is clearly only present in the expanded state and should therefore be merely a watery medium; to postulate the presence of an "amorphous matrix" between the fibrils (e.g. in REYNOLDS, 1989: 13) is superfluous.

A more or less severe decrease in wall thickness of apical domes during the ascus ontogeny can be observed in dead asci (and less pronounced in living asci) (BARAL, 1987b: 124). This effect was often misinterpreted to be a result of increased turgor during maturation (e.g. by HONEGGER, 1983: 63, figs. 3a→b, 4b→c, all figured asci were killed by acrolein-glutaraldehyde/osmium-fixation). The difference in thickness is indeed also present in ruptured asci in water mounts. Thus, changes in the fibrillar compactness, which may represent different degrees of polymerisation should have occurred during maturation. Reported variation in thickness of the tholus in dead asci of **Tephromela aglaea** as depicted by HERTEL & RAMBOLD (1985) should partly be referred to this effect. My experience is that living asci show much less variability in their apical structures compared to dead asci in any group of Ascomycetes. Nevertheless, I concur with the common practice of considering the expanded apical apparatus more useful for taxonomic purposes because the amyloid structures are too compressed in the living state to be able to see the important details. The morphology of the living apical apparatus should, however, be simultaneously studied.

Since the observed decrease in wall thickness during maturation of the ascus is the result of a reduced hydration of the microfibrils, the theory of CHADEFAUD (1942: 65) and BELLEMÈRE & HAFELLNER (1982: 272) claiming resorption of wall material during the "regression" of the thickened wall layers is also superfluous.

Variation in thickness is sometimes increased in dead asci by mechanical pressure of the spores against the endotunica, especially in asci with expansible lateral walls. Thus, **Tympanis**, for example, exhibits thick apical and lateral walls in young dead, or in discharged asci but thin lateral walls in mature, non-discharged, dead asci (FIG. 10b) as a result of mechanical pressure of the numerous ascoconidia.

Many accounts of the fissitunicate ("bitunicate") ascus indicate that the wall in the upper region, especially of the immature ascus, is thickened. In the living state, however, fissitunicate asci are thin-walled at any stage of development (DE BARY, 1887: 95; HOGGAN, 1927; KERR, 1961: 475f.). The *banded pattern* or *accordion-like arrangement* of the microfibrils, considered to characterize the non-discharged fissitunicate ascus (REYNOLDS, 1971: 248, fig. 7; MÜLLER, 1981; PARGUEY-LEDUC & JANEX-FAVRE, 1982: figs. 15-20; BELLEMÈRE & HAFELLNER, 1982: fig. 6; BELLEMÈRE & al., 1986: fig. 3), occurs logically only in dead asci (FIG. 7, the fibrils are not seen with the LM). The theory of a "reorientation" of the microfibrils from a banded to a parallel pattern during elongation of the endotunica (e.g. in REYNOLDS, 1971: 254; 1989: 14) is thus superfluous.

Many attempts to reconstruct the process of spore discharge start with the false assumption that the endotunica is thick-walled prior to bursting of the ascus, or that it at least swells prior to discharge. In the Leotiales, numerous observations of spore discharge from asci in water mounts confirm that the apical dome is thin-walled as the asci explode (BARAL, 1987b: 128). The possibility of monitoring the delayed process of successive spore discharge from fissitunicate asci already led

PRINGSHEIM (1858) to the discovery that swelling of the endotunica occurs only after the last spore has been forcibly ejected.

A theory advocated by DUGHI (1957) and CHADEFAUD (1942: 59; 1973: 135f.) claims that tholi in asci of Lecanorales play a role in drought tolerance. The authors correctly refer the variation in thickness to a varying degree of hydration of the ascus wall, but thought that this variation depends upon the atmospheric humidity. The latter is not true: by mounting in oil, I found the endotunica in living asci of **Lecanora conizaeoides** to be always compressed, whether the asci were fully or partly hydrated, or dehydrated by air-drying. Furthermore, remarkable drought tolerance and longevity was found to occur also in asci devoid of wall thickenings (see also chapter 9).

Mounting fresh apothecia in concentrated ethanol, or air-dried apothecia in oil, showed dehydrated and thus thin walls in the ascus apex even in dead asci. This effect was discussed by STEINER & PEVELING (1984: 784) for ascospores with expansible septal walls. In TEM-preparations, different methods of fixation often result in different degrees of expansion (see below).

What is the biological sense of expansible inner ascus wall layers? DUGHI (1957: 13) listed 7 hypothetical functions of the tholi in Lecanorales. From the preceding, I am forced to assert that none of these hypotheses can be maintained. The process of expansion through water absorption prior to discharge must be considered an anomaly in all Ascomycetes with expansible layers: expansion occurs when asci die but never in living asci under natural conditions. Active and complete discharge from dead asci, as indicated by DUGHI, was never observed. Layers capable of expansion (= swelling in thickness) are, however, capable of enormous extension (= elongation in longitudinal direction) directly prior to spore discharge, especially in the fissitunicate ascus (DE BARY, 1887: 96; KERR, 1961: 475; BARAL, 1987b: 128). Apical rings are thereby everted prior to discharge (BARAL, 1987b: figs. 1, 7). Extensibility is presently the only function of expansible layers that I am able to accept.

That the expanding wall phenomenon in asci has almost completely been forgotten in the last decades is obvious from STEINER & PEVELING's (1984) recent account. The authors reported a similar effect for inner wall layers of the thick-walled septa in ascospores of **Caloplaca/Xanthoria** (FIG. 5), and correctly refer the swelling of the septum to the death of the spores. They do not mention, however, that thickened walls of asci show the same effect. I have found comparable expansible septa in (especially immature) spores of **Physcia**, **Rinodina**, **Massaria anomia** and **Nimbomollisia**. NANNFELDT (1983: 297) characterized the latter genus by spores having thick and "refractive" septa (*septo mediano crasso, valde refringente divisae*). Yet, only dead spores have thick septa (FIGs. 12b, 13b) while living spores show thin-walled septa (FIGs. 12a, 13a). Expansible wall layers are said to occur also in spores of Endogonaceae (SPAIN, 1990: 72, induced by acidic mountants), and in septal structures (dolipori) of Basidiomycetes (HOCH & HOWARD, 1981; LÜ & MCLAUGHLIN, 1991). The latter observed under the TEM, that

the height of the pore swellings by freeze substitution was 100-184 nm (figs. 2, 4-10) but 370-475 nm by conventional chemical fixation (figs. 3, 11).

5.c. Contrast of cell walls and mucilaginous sheaths

The loss of contrast of the endotunica during the death of the ascus was already mentioned above. The contrast of the spore wall inside asci becomes also often strikingly faint if asci and spores are killed by KOH, CB etc. This is due to the fact that, in the living state, the spores are surrounded by ample transparent (non-refractive) water of the vacuole(s). Therefore, the refractive spore wall markedly contrasts with the surrounding water. In the dead state, however, most of the water has escaped from the vacuole(s), and the spores are now embedded in the dead epiplasm which has a refractive index comparable to that of the spore wall. Dead ascospores inside dead asci of Leotiales can therefore often only be recognized from their LBs (in KOH) if LBs are present, while the spore wall remains quite invisible.

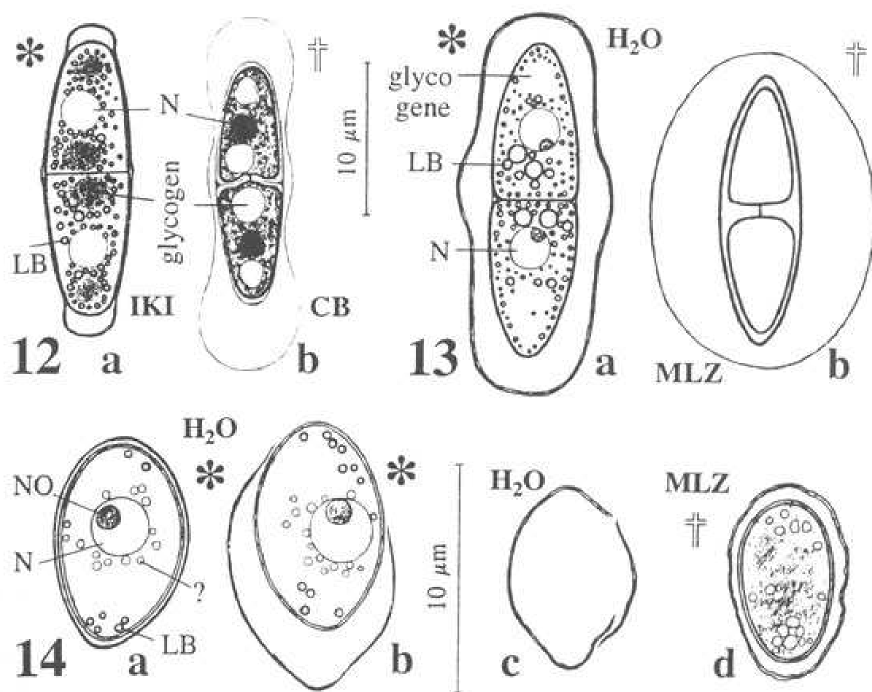
In the species of **Calycellina** with 4-spored asci in the mature stage, 8 spores are formed at first but 4 spores early abort and collapse (BARAL, 1989b). The remaining cell walls of the collapsed spores are clearly visible within living asci (FIGs. 19, 20) but entirely indiscernible in asci from herbarium material. Therefore, this interesting situation has not been reported by other authors.

Coalesced LBs may simulate septa (FIG. 22). In living multiguttulate spores true septa are not easily seen but recognizable by slight constrictions of the spores at the septa and by the nucleus in the middle of each cell (FIGs. 1a, 2ab). True septa often become more obvious when mounting in MLZ, CB etc. (FIG. 7b), but then the asci are killed and, therefore, the degree of maturity of the spores, i.e. the number of septa in the mature spore, is not further recognizable (see chapter 8). BOUDIER (1886: 143f.) recommended to stain *in statu vivo* by IKI in order to see septa more clearly.

The presence or absence of croziers on the ascogenous hyphae, recently re-introduced by HUHTINEN (1990a: 66) as a very important taxonomic character in **Hyaloscypha** and allied genera, is rapidly seen *in situ* in sections through living young apothecia in most taxa of Leotiales (avoid pressure on the cover slip). In dead hymenia, however, the feature is often indiscernible when water, MLZ or CB is used. Mounting in Congo red (gently heated) as HUHTINEN recommended (in litt.), or in KOH as I prefer in the case of herbarium material, usually allows this feature to be seen when separating the tissue by pressure, but it is very often highly time-consuming to be certain about it. The feature has a high significance for the delimitation among the species of Leotiales: 77% of 767 species so far studied by me have croziers but 22% have not. About 1% seem to be variable.

Mucilaginous appendages or sheaths of spores are often overlooked in herbarium material. Sheaths swell considerably after spore discharge (INGOLD, 1978; BRUMMELEN, 1967: 39), or if asci are killed together with their spores, rarely

even within the living ascus, and thereby lose refractivity and contrast (FIGs. 12 a→b, 13 a→b). Obviously, a delicate semipermeable membrane covers the exterior of the mucilage. These sheaths have wrongly been thought to supply the internal pressure for spore discharge by several workers. Numerous observations on living material proves, however, that the sheaths are always dehydrated and appressed to the spore wall shortly before and during spore discharge. In dead non-discharged asci they are strongly swollen and fill the space between spore and ascus wall. In some taxa these sheaths stain violet in CRB.



FIGs. 12-13. Spores of **Nimbomollisia** observed in the living versus dead state. The expansion of the mucilaginous sheath occurs when spores are killed but also in the living state: sheaths are compressed inside living asci and expand sooner or later when spores are discharged or when asci die prior to discharge. Note shrinkage of spores and thickened septum. **FIG. 12. N. eriophori, FIG. 13. N. melatephroides.** 2000x.
FIG. 14. Non-mucilaginous sheath (perispore?) in spores of **Ciboria caucus**. The sheath bursts and finally separates due to imbibition of the living spore after discharge (a→b→c). **a.** spore inside living ascus; **b.** spore immediately after discharge; **c.** completely separated sheath; **d.** anomalous separation from the spore wall without bursting due to shrinkage of the spore after killing by MLZ. 3000x.

Other taxa have very thin sheaths of mucilage on the ascospores which become only visible in the case they are stained by CRB showing a weak or deep reddish-violet. This feature of high taxonomic value occurs, e.g., in the species of **Pezicula**, in several species of **Calycellina** (BARAL, 1989b: 212) and **Ombrophila**, in **Calycina alniella**, **Hyaloscypha aureliella**, and **Durella connivens**. **H. aureliella** is easily distinguished from **H. britannica** var. **britannica** by this reaction. The reaction seems so far unreported for the Leotiales. GRUBE & HAFELLNER (1990: 307) found a red-violet stain of the spore wall in some species of **Zwackhiomyces** using aqueous methylene blue. I found this dye to give only a weak bluing to spores reacting deep violet in CRB. CB gave always negative results due to the presence of lactophenol as did CRB + lactophenol. This type of reaction should therefore be distinguished from blue (*cyanophilous*) reactions in CB and is better termed *metachromatic* (see SINGER, 1986: 80, *metachromatism with cresyl blue*).

A delicate, scarcely mucilaginous but inelastic, unstainable sheath (perispore?) was found to occur very frequently in spores of Leotiales. This sheath bursts by separating from the true spore wall after discharge, due to release of ascus turgor and therefore increase in the spore volume (FIGs. 14, 22a, 35a). The spore thereby completely slips out of its sheath. Such sheaths appear not to have been reported in this order by other authors, probably because they are very difficult to see in herbarium material.

6. ALTERATIONS IN THE CELL CONTENTS

Fungal cells often contain more or less refractive cytoplasmic inclusions. BOUDIER (1886: 143; 1907: 28; 1914: 51) was one of the few taxonomists who have emphasized the fact that, in the living state, these inclusions show a strikingly stable and regular image which often serves as an *excellent criterion* in the taxonomy of Ascomycetes. My observations on numerous species confirm BOUDIER's statements. The delimitation among many taxa becomes decidedly facilitated if guttules inside living spores and paraphyses are used as additional features. Dead cytoplasm, however, shows highly variable patterns; its morphology depends furthermore on the mounting medium, and many types of inclusions can no longer be discerned. The variable morphology of the cell contents in different states and development stages observed in water mounts misled many researchers (e.g. KILIAS, 1981: 269) to disregard cytomorphological features and to prefer mounting media which clear the contents and kill the cells. Workers accustomed to studying dead spores sometimes believe the interior of the living spores to represent an anomaly: BENKERT (1976: 632), for example, assumed that the multiguttulate (living) spore in **Melastiza chateri** (FIG. 16f) is an anomalous state while the biguttulate (dead) spore (FIG. 16g) represents the normal case.

Refractive cell inclusions are best visible in the fully hydrated cytoplasm (in water mounts) using bright field optics. The contrast of the inclusions depends on

various circumstances: it decreases with increase of the magnification used, and with higher refractive index of the cytoplasm, e.g. by natural dehydration of the spores during maturation in many Pezizales. Applying phase-contrast proved only superior with large cells, but seems useful when mounting in a plasma albumin medium which has a high refraction but a low osmosity (BARER et al., 1953).

6.a. Lipid bodies (LBs, "oil drops")

Lipid forms globose refractive bodies of about 0.2-10 μm diam. within the cytoplasm outside the vacuoles. In germinating spores the lipid usually disappears and serves as an energy and carbon reserve (STEINER, 1957: 242; SUSSMAN & DOUTHIT, 1973: 315; WEETE, 1981: 465). For a review of LBs in plant cells see GURR (1980) and WANNER et al. (1981), for fungi see HESS (1981) and WEETE (1981).

Recognition:

Recognition of lipid can be made by two tests (BARAL, 1989a): (1) 1-5% KOH does not dissolve LBs when added to living or dead cells (LBs remain visible in full strength, even after boiling); (2) staining with CRB is negative while CRB_A stains LBs within dead cells yellowish-amber to deep copper-orange.

Lipophile dyes are commonly used to give more or less specific stains for fatty matters (see KIRK, 1966: 87ff.). Most of these tests are lethal to the cells. I have tested Sudan III in lactic acid in several species: a distinct reddish stain of the LBs was rapidly obtained especially after heating the slide while CRB_A gave the characteristic amber stain. KIRK (l.c.) recommended two tests (using benzapyrene-caffeine, and neutral red) for staining LBs *in statu vivo* by fluorescence.

KORF & ERB (1972) and KORF (1977) found **Trichophaeopsis bicuspis** to differ from **Trichophaea** by ascospores with "non-oleaginous, somewhat resinous inclusions" instead of LBs since the inclusions failed to absorb oil stains such as Sudan IV. My material of **T. bicuspis** showed two polar LBs 3.5-4 μm in size. Indeed, the whole spore content remained unstained by CRB_A or Sudan III for hours, even after boiling, except for immature, and ruptured mature spores, where the LBs stained amber in CRB_A and red in Sudan III. Thus, negative result is clearly due to the impermeability of the thick wall of the mature spore to these dyes.

Formation:

LBs in ascospores either originate from minute precursors (FIGs. 15b, 16b; BARAL, 1984: fig. 7) or are already present in the cytoplasm of the ascus before the spores are formed (FIG. 42a). LBs increase during sporogenesis (without fusing) to a very different size, depending on the species (FIGs. 15, 16, 42).

Distortion:

Coalescence (fusion) of several LBs is frequently found in dead spores in a fresh ascocarp (and also in other groups of fungi with spores with high lipid contents), and is considered an anomaly caused by damage to the limiting membrane of the single LBs (HEINEMANN, 1956: 40ff.; STEINER, 1957: fig. 18; CUNNELL, 1959: 465; FREY-WYSSLING & MÜHLETHALER, 1965: 168ff.; KIRK, 1966: 70; GURR, 1980; WANNER & al., 1981). LBs may thereby lose their spherical shape by forming irregular aggregations with the surrounding cytoplasm (FIGs. 1 a→d, 2 ab→cef, 7 a→b, 16 f→g, 21 a→b, 22 a→b; HUHTINEN, 1990a: fig. 175c, H_2O→MLZ). STEINER pointed out that such state-dependent effects were the reason why cells of **Saccharomyces cerevisiae** have erroneously been thought to vary considerably in oil content (indistinctly multiguttulate *in statu vivo*, one large distinct drop *in statu emortuo*). According to KIRK (l.c.), *no fixatives entirely prevented guttular oil from spreading throughout the spores.*

On the other hand, coalescence does (? regularly) not occur if spores have died in the dried state whilst lying in the herbarium: spores inside asci exhibited undamaged guttule patterns even in about 100 years old dried material when studied in KOH.

Coalescence of the LBs can be induced within a few seconds when killing multiguttulate spores by adding ethanol, HCl (STEINER, l.c.), CB or MLZ to the water mount, or can be observed in spores in water as they die. Thereby, coalescence occurs some seconds or minutes prior to the loss of cell turgor (see FIG. 2c where the small LBs have coalesced in the living spore). The contrary process, the formation of many small drops from one large drop, though reported by researchers, was never observed, either by me or by CUNNELL (l.c.). Note, however, that multiguttulate immature spores may regularly develop into oligoguttulate mature spores, for example in the genus **Octospora.** This effect occurs without fusion of LBs: only one or few of the small LBs grow to a large size so that the remaining small ones are overlooked when viewed at a low magnification. Such a case is figured by JOHNSON (1963: pl. 33, figs. 1-6) for living asci of **Ceriosporopsis.**

A few seconds after coalescence takes place, the lipid often disappears optically (in MLZ, CB, or water): due to (1) the loss of cell turgor and therefore dehydration of the cytoplasm (increase in refraction, FIGs. 15g, 16g), (2) the fact that in shrunken spores with a high relative lipid content the lipid continuously fills the whole cell (FIGs. 2d, 5b), or (3) a high refractive index of the employed mountant which imbibes the cytoplasm around the LBs (FIGs. 8b, 12b, 13b). These effects explain why LBs in spores with rich lipid contents are frequently figured "empty" in many publications (without comment), provided that the contents were not omitted intentionally. HERTEL (1967: 15), KORF (1977), SPOONER & DENNIS (1985: 298) and SVRČEK (e.g. 1989: 72) reported guttules (certainly LBs) "disappearing" from spores after prolonged preservation in the herbarium, or when mounted in MLZ or CB. RAMSBOTTOM (1916) already drew attention to the "disappearance" of guttules in spores when mounted in glycerol: *In no case have guttulae been*

observed in the collection of Discomycete slides preserved in the National Herbarium. The lipid reappears, however, with more or less strong contrast even in old herbarium specimens if the spores are mounted in 1-5% KOH (KORF, l.c., obtained this effect using KOH-phloxine-glycerol). A varying refractivity of the cytoplasm is also the reason why DODGE (1957) reported the LBs to "disappear" reversibly during DBB-formation (FIG. 11 g→h).

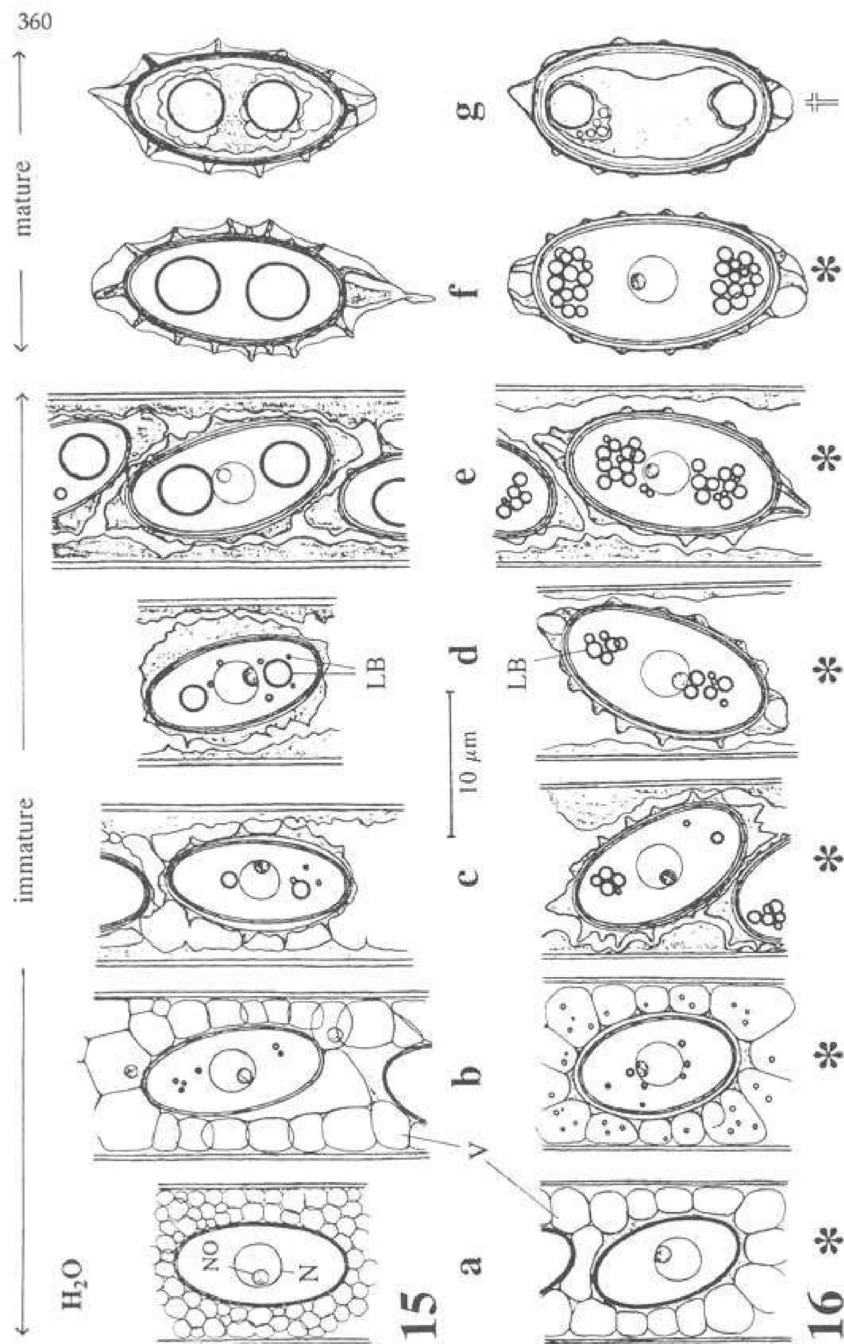
When living spores pass into the germination phase, the lipid is actually broken down and used for energy production and synthesis of new cellular material (DE BARY, 1887: 113; GARRAWAY & EVANS, 1984: 227). It is therefore very important for the purpose of taxonomy to use only mature spores for the study of spore guttulation (see chapter 8).

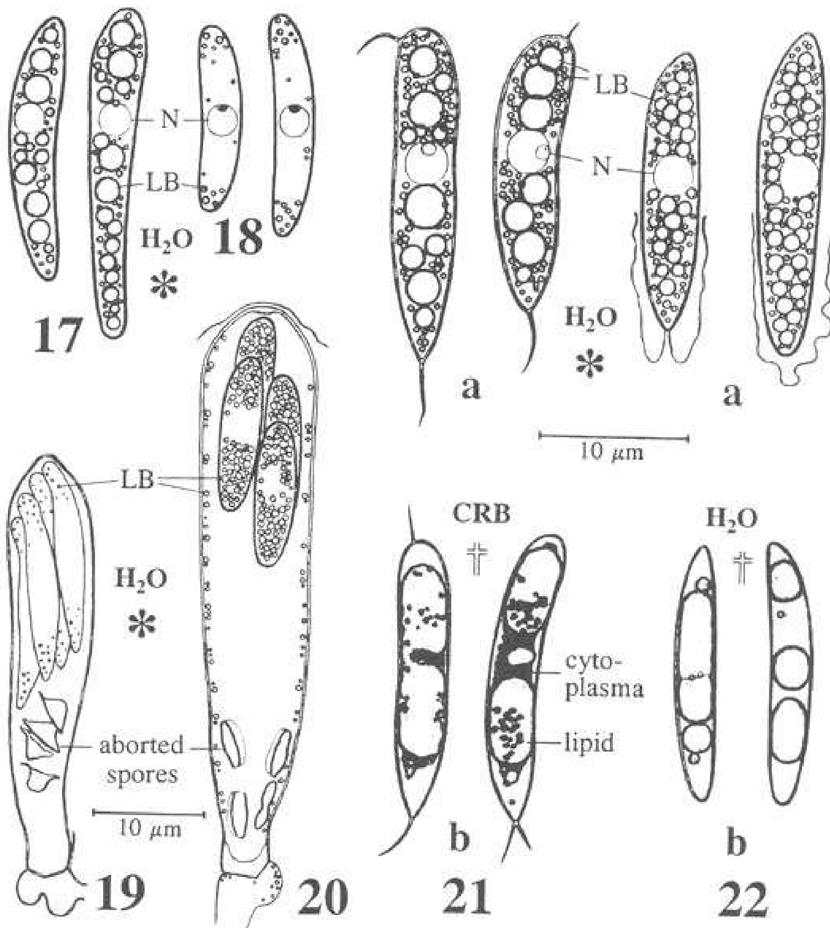
Taxonomic value:

BOUDIER (1907: 28; 1914) drew attention to the taxonomic importance of LBs in ascospores and regretted the fact that workers have often ignored the guttules in their descriptions. LE GAL (1947) gave a survey of the various types of guttular patterns in the spores of Pezizales. HERTEL (1967: 15) emphasized the taxonomic use of guttules in spores of Lecanorales. KARSTEN (1871) already noted spore guttulation in many species. Many other authors included this feature in their descriptions but few took influences of their methods into account. *The importance of fresh material for species diagnoses, especially for noting ascospore guttulation, cannot be overstated* (HARRINGTON, 1990: 436). Since lipid serves as a nutritive substance, differences among related taxa in the amount of lipid within mature spores seem to reflect differences in ecological adaption in regard to spore germination.

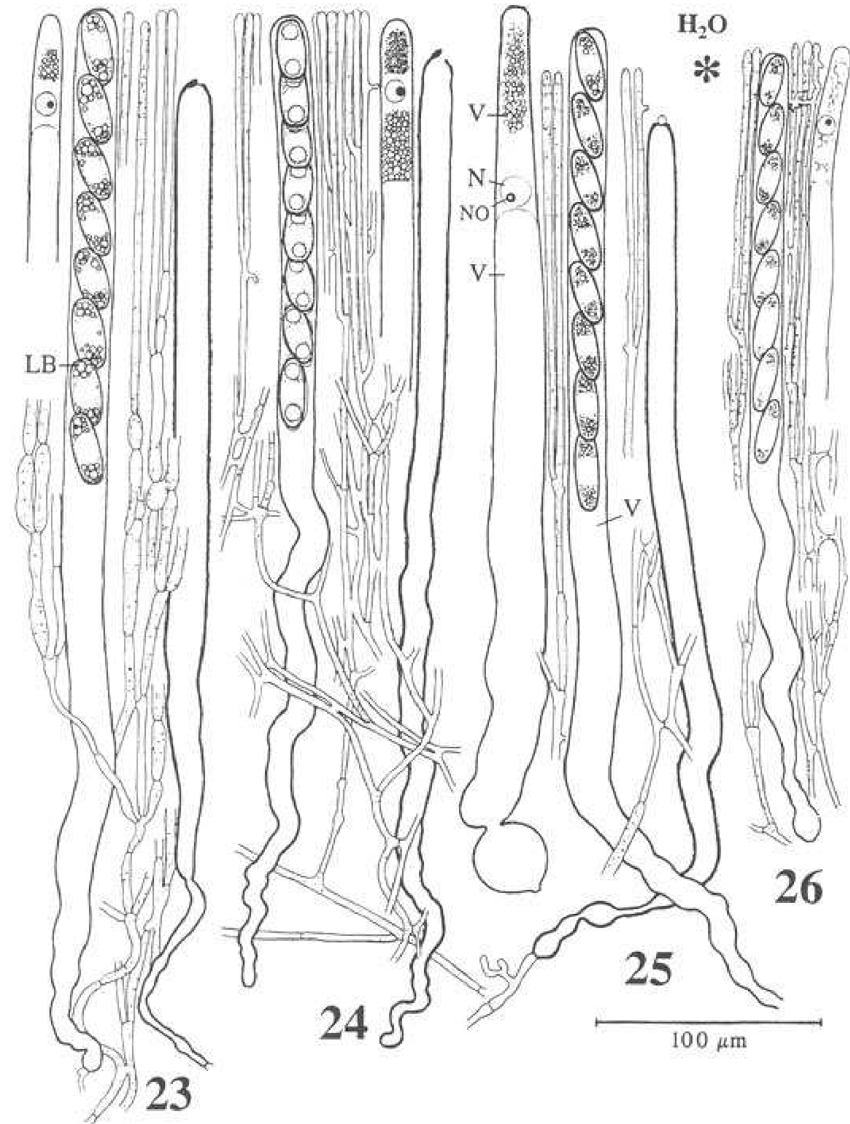
In mature ascospores the total amount of lipid often severely varies between closely related species (**Calycellina**, FIGs. 19-20, **Lasiobelonium**, FIGs. 17-18). Similarly, **Morchella**, **Cheilymenia**, **Tricharina** and **Peziza** p.p. ("**Aleuria**") differ from **Helvella**, **Scutellinia**, **Trichophaea** and **Peziza** p.p. ("**Galactinia**") (BOUDIER, 1885: 101, 104, 105; 1914: 54; KORF, 1977; BARAL, ined.), or **Ciboria** from **Rutstroemia** (BARAL & KRIEGLSTEINER, 1985: 10, 19) in the absence versus abundant presence of LBs in the spores. HUHTINEN (1990a) considered **Calycellina lutea** a possible synonym of **C. lachnobrachya** (as "**Phialina**"). The spores of **C. lutea** are misleadingly depicted eguttulate in the original description. Yet, these contain many LBs in contrast to **C. lachnobrachya** with a low lipid content (FIGs. 19-20). Likewise, **Lasiobelonium variegatum** and **L. corticale** have often been confused because, besides other features, the striking difference in the lipid content was overlooked (FIGs. 17-18). Numerous other such "critical" species can be separated on the basis of the quantities of lipid.

CHADEFAUD (1969: 195) characterized different orders of pyrenomycetes by the lipid content in ascospores (*type «pauvre»* versus *type «riche»*). WEETE (1981: 464) stated that the *lipid content of spores of most fungi generally ranges between*





FIGs. 15-22. Four taxon pairs, easily distinguished in the living state by the lipid content of the mature spores (high versus low content: FIGs. 17-20; few large versus many small LBs: FIGs. 15-16, 21-22). Note that (1) coalescence of LBs obscures the distinctive features (FIGs. 15/16 f→g; 21/22 a→b), (2) LBs are absent in the first stage of ascosporogenesis in certain taxa (FIGs. 15a, 16a), but present in others (FIG. 42 abc). **FIG. 15. Aleuria aurantia, FIG. 16. Melastiza chateri** (1500x); **FIG. 17. Lasiobelonium corticale, FIG. 18. L. variegatum** (2000x); **FIG. 19. Calycellina lachnobrachya, FIG. 20. C. lutea** (1500x); **FIG. 21. Hymenoscyphus scutula, FIG. 22. H. consobrinus** (2000x, the dead spores were found in the squash mount together with the living spores). (FIGs. 19-20 from BARAL 1989b).



FIGs. 23-26. The four European species of **Sarcoscypha** distinguished, besides other features, by the size of the lipid bodies in the ascospores (from BARAL, 1984: fig. 4, this figure was unintentionally issued only with its upper half, and is here reproduced in its full extent). **FIG. 23. S. austriaca** (neotype), **FIG. 24. S. jurana, FIG. 25. S. coccinea** (neotype), **FIG. 26. S. macaronesica** (holotype). (300x).

5 and 17% of their dry weight, but spores of some species such as rusts may contain up to 35%. I have estimated the total volume of lipid in relation to the volume of the living hydrated spore by the area fraction of the LBs in optical section which is proportional to their volume fraction (WEIBEL et al., 1966). I use the following linear scale: 0 = devoid of lipid; 5 = maximum lipid content. In the ascospores of 815 tested species of Leotiales, these categories were represented in quite equal frequencies, with a slight maximum towards the lower contents: 0-1: 21%; 1-2: 25%; 2-3: 18%; 3-4: 16%; 4-5: 20%. Usually, there is little variation within a species if only living mature ascospores are taken into account.

Differences in the lipid content of asci prior to spore formation are outstanding between certain taxa although, in these case, the spores are finally always rich in lipid: asci of **Pezicula** (FIG. 42) and **Pachyella babingtonii**, for example, have high contents in the stage around meiosis while those of **Sarcoscypha** (FIGs 23-26), **Otidea** and many studied Humariaceae (FIGs. 15a, 16a) have very low contents.

The size of the single LBs in spores (few large versus many small LBs) is a further, usually consistent feature which supports delimitation among many species (FIGs. 15f-16f, 21a-22a). In **Sarcoscypha** (FIGs. 23-26), the size of the larger LBs allows the delimitation among species (BARAL, 1984). Living spores have been studied from 26 collections of **S. jurana**, 30 of **S. austriaca**, 14 of **S. coccinea**, and 4 of **S. macaronesica**. The features were consistent: The first species showed the largest LBs and occurred only on **Tilia**, the second had medium-sized LBs and produced conidia on the ascospores, the two latter differed by small LBs and obtuse spores.

According to DENNIS (1978: 400), Dothioraceae and Dothideaceae usually differ in multiguttulate versus uni- or biguttulate cells of ascospores. HEINEMANN (1956: 41ff.) found **Saccharomyces cerevisiae** to differ *in statu vivo* by cells with many LBs from other Endomycetales having cells with one large and a few small LBs. The Helvellaceae (**Discina**, **Helvella**, **Rhizina**) are characterized by spores with usually one large central LB with some accessory LBs while **Gyromitra** (Morchellaceaea) is strictly biguttulate (BENEDIX, 1966: 360, figs. 1, 2, 4, 5; BARAL, ined.). HERTEL (1967: 15, pl. 15) used different amounts and patterns of lipid in living ascospores of **Lecidea** as a character on the specific level. In the genera **Lecanora** and **Diaporthe** I observed species with multiguttulate spores (FIG. 9) and other species differing by strictly biguttulate spores.

6.b. Refractive vacuolar bodies (VBs)

Normal living fungal vacuoles are totally non-refractive and can be detected under the LM as transparent ("empty") regions within the cytoplasm (FIGs. 27, 28, 31, *V*). CRB or CRB_A gives a homogeneous violet stain to the vacuoles (typical for the single large vacuole in mature asci), or mostly induces flocculation of blue-violet MCs in the vacuole sap (FIGs. 4, 30). A phenomenon characteristic of a

major part of the Leotiales (but absent in the other part) is, however, the presence of a specialized type of vacuole side by side with the normal type (FIGs. 27-29, 32-33, *VB*; HUHTINEN, 1990a: fig. 255c, H_2O). This special type contains a colloidal substance of a more or less high refraction within the tonoplast, with the vacuoles appearing "full". Here, CRB never induces the formation of MCs but rather stains the bodies in a homogeneous turquoise-blue. GUILLERMOND (1941: 161, 181ff.) and BANCHER & HÖFLER (1959: 152) described similar vacuoles occurring in vascular plants as opposed to normal vacuoles: highly refractive, more acid, staining *in statu vivo* blue or green by CRB, reducing osmic acid, rich in phenolic compounds (tannin).

Such *refractive vacuolar bodies* (*VBs*), as I term this type of inclusion, occur predominantly towards the surface of the ascocarp: in the top cells of paraphyses, outer excipular cells, or basal part of hairs (FIG. 32). BELLEMÈRE (1958) described them in **Cyathicula coronata** as *granulations réfringentes, brunâtres après coloration par la réaction de A. Prenant* in paraphyses and cortical hyphae. Due to their refraction they have mostly been misinterpreted as lipid bodies (see BARAL, 1989a: 120; 1989b: 225). In certain genera, IKI or MLZ give a reddish-brown reaction to VBs (see BARAL, 1987a: 424; SVRČEK, 1989: 73; HUHTINEN, 1990: 71, as *golden*). The IKI reaction is stable while the MLZ reaction disappears within about 1/2 min. In many mollisiaceous fungi, KOH provokes a deep sulphur-yellow reaction with the VBs (see chapter 7). Hydrophilous (mainly yellow) pigments sometimes occur in VBs (FIG. 32). On the other hand, oxidative color changes to yellow or reddish-brown often occur when cells are injured (this supports the idea that phenolic compounds are involved). Therefore, what HUHTINEN (1990a: 71) described as *yellow pigment* mainly from herbarium specimens and found to be diagnostic for **Calycellina** (as "**Phialina**") is just the same as what I term *VB*. I have found VBs also in vegetative surface cells of Basidiomycetes (**Clavariadelphus**, **Ramaria**). The greenish-blue (turquoise) color of VBs, obtained by staining *in statu vivo* with CRB or toluidine blue, is a purely metachromatic effect and does not indicate a more acid pH because, in the case of toluidine blue, changes in the pH do not affect the color of the dye. According to HARMS (1965, II: 19), basic dyes do not permit a clear evaluation of the pH inside the vacuole.

Recognition:

Delimitation from LBs can be made by two tests: (1) VBs are dissolved instantaneously (complete optical disappearance) by 1-5% KOH (but not by even strong acids) when added to living cells (FIG. 33 a→b, c→d); (2) staining *in statu vivo* with CRB or CRB_A always gives a strong pure turquoise-blue (metachromatic) color to hyaline VBs (BARAL 1989a: 121) within a few minutes.

Numerous taxa have been tested, but only a single exception was found: in species of **Symphyosirinia**, the large conidia of the anamorph are completely filled with strongly refractive VBs which stain deep turquoise in CRB but are not dissolved when killed by a strong KOH-treatment.

Formation:

The refractive substance becomes apparent inside young small vacuoles, e.g. at the tip of paraphyses, as a colloidal solution. During development VBs increase in size. Two main types can be distinguished: (1) multiguttulate type (FIGs. 27, 33): many small vacuoles are formed at the beginning; these later grow in size while the substance sooner or later precipitates within the single vacuole by forming many small globose VBs which show Brownian movement in the transparent vacuole sap (lower part of paraphyses); (2) elongate type (FIGs. 28, 29, 32): few large vacuoles are formed in which the substance remains colloidal in the living state. Intermediate types also occur representing type (1) but with VBs remaining permanently colloidal in the living state.

Due to the mentioned ontogenetic changes in size and shape of the VBs, there is often a slight variation from young to mature apothecia. The feature is, however, highly stable in most taxa if only adult living cells are considered.

Distortion:

Lethal mountants (MLZ, CB, KOH) destroy VBs resulting in their complete invisibility (FIG. 33; see HUHTINEN, 1990a: fig. 255, H_2O vs. MLZ). KOH probably provokes hydrolysis of the refractive molecules. VBs may also disappear within less than 1 sec. if the cells die during examination of a water mount, possibly due to a raised pH when the tonoplast bursts. Herbarium material often lacks any remnants of VBs or shows irregular bodies with often altered pigmentation which are then KOH-insoluble (FIG. 1f; HUHTINEN, 1990a: figs. 212-261, illustrated as black intracellular spots).

Taxonomic value:

VBs in paraphyses and in cortical cells or hairs are of importance in the following examples: **Cyathicula** is easily distinguished by strongly refractive VBs of the multiguttulate type (FIG. 27) and blue reacting apical rings of the **Hymenoscyphus**- or **Bulgaria**-type from **Allophylaria**, which has elongate VBs (FIG. 28) and red reacting apical rings of the **Laetinaevia**-type (BARAL & KRIEGLSTEINER, 1985: 108; BARAL 1987b). Variation in the type of VBs was only seen in 2 of the 13 studied species of **Allophylaria** (showing a tendency to multiguttulation), and in 1 of the 20 studied species of **Cyathicula** (showing a weak refraction). Due to lack of fresh collections there is still no information available about the paraphysis content in the type species of **Crocicreas** which would help in clarifying its relation to **Cyathicula** which was placed in synonymy of **Crocicreas** by CARPENTER (1981). Whilst monographing this large genus, CARPENTER almost completely ignored VBs (but several reports of yellowish contents and a darker stain of the paraphysis plasma by CB or MLZ give indirect indication of their presence). He therefore classified a typical **Allophylaria**, **A. subhyalina**, in **Crocicreas**.

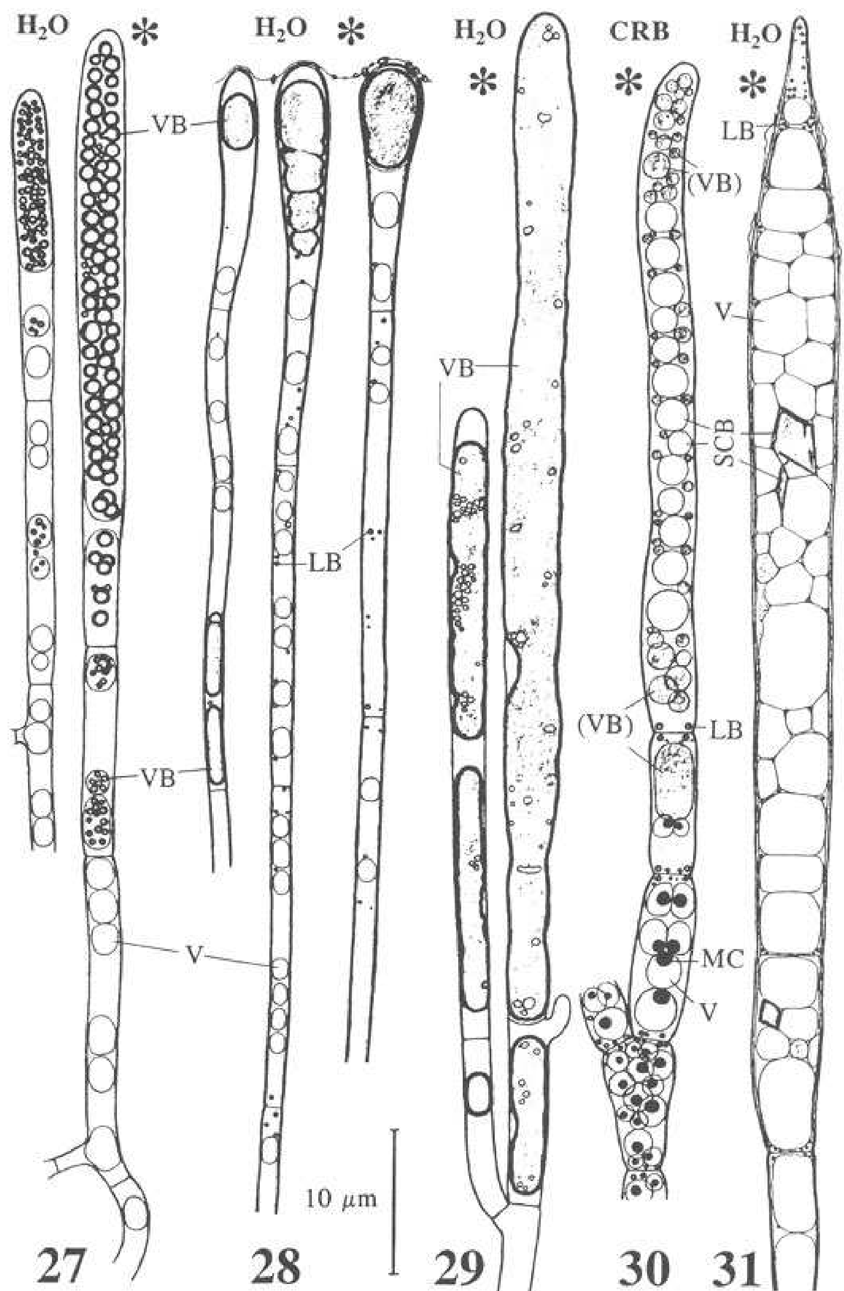
A similar case probably requires a generic split within the genus **Bisporella**: **B. pallescens** and **B. subpallida** have multiguttulate VBs and apical rings of the **Hymenoscyphus**-type, while **"B." citrina**, **"B." lactea**, **"B." sulfurina** and **"B." scolochloae** have elongate VBs (the two former species have apical rings of the **Laetinaevia**-type, the two latter are IKI-).

Mollisia/Tapesia (FIG. 29) and **Calycellina/Phialina** (FIG. 32) are characterized by highly refractive, more or less elongate VBs while **Pyrenopeziza/Pirottaea** (FIG. 30) and **Hyaloscypha** show globose VBs of very low or vacuoles lacking refraction (BOUDIER, 1885: 119; BARAL & KRIEGLSTEINER, 1985: 35; BARAL, 1989b; HUHTINEN, 1990a: 71). The observed variation was as follows: in **Mollisia** s.l., 44 studied species showed medium to strong refraction while 10 showed low or lacked refraction; in **Pyrenopeziza** s.l., all 27 species showed low or lacking refraction. All 30 studied species of **Calycellina** s.l. had medium to strong refraction but 12 out of 13 studied species of **Hyaloscypha** showed no refraction while only 1 (**H. secalina** var. **paludicola**) differed in having strongly refractive VBs. Refractive VBs of the multiguttulate type are typical in the "**Hysteropezizella**-complex".

In **Lachnum** s.str., 18 out of 46 studied species showed multiguttulate VBs (FIG. 33) whilst 26 lacked VBs (2 are variable), and it is the VBs which are responsible for the reddish color change of the white apothecia (BARAL & KRIEGLSTEINER, 1985: 73). The apices of **Botryotinia** paraphyses (5 studied species) contain ochraceous refractive VBs which are absent in **Sclerotinia** (3 species) and **Myriosclerotinia** (2 species). This seems an unpublished feature which supports the justification of the genus **Botryotinia**, recently put back in synonymy of **Sclerotinia** by SPOONER (1987: 202ff.), who regarded the difference in conidial states as no more than of subgeneric value. **Discina ancilis** and **D. gigas** show ochraceous, strongly refractive VBs of the multiguttulate type (FIG. 40a) which **Gyromitra esculenta** lacks.

Distinct VBs rarely occur in spores. They are characteristic, however, of ascospores of certain taxa, a hitherto overlooked phenomenon, e.g., of most species of **Orbilia** (FIG. 37, showing a highly characteristic shape), of **Hymenoscyphus** cf. **sazavae** (FIG. 35a), and of **Tubeufia paludosa** (FIG. 34c). Vacuoles of low refraction often occur in ascospores of Leotiales (FIG. 36; CHADEFAUD, 1969: 191, figs. 8a, 9b, 10b).

FIGs. 27-31. Five species differing by the contents in their paraphyses (refractive versus non-refractive, multiguttulate versus elongate, restricted to the tip of paraphysis versus reaching down towards the base). Note difference in refraction between non-refractive vacuoles (*V*) and strongly refractive vacuolar bodies (*VB*) (in FIG. 30 the VBs have a very low refraction). The metachromatic corpuscles in FIG. 30 are produced through staining *in statu vivo* with CRB, all other structures are present without any treatment. **FIG. 27. "Conchatium" fraxinophilum** (= **Cyathicula fraxinicola**). **FIG. 28. Allophylaria nervicola**. **FIG. 29. Mollisia** spec.. **FIG. 30. Pyrenopeziza petiolaris**. **FIG. 31. Brunnipila clandestina**. 2000x.



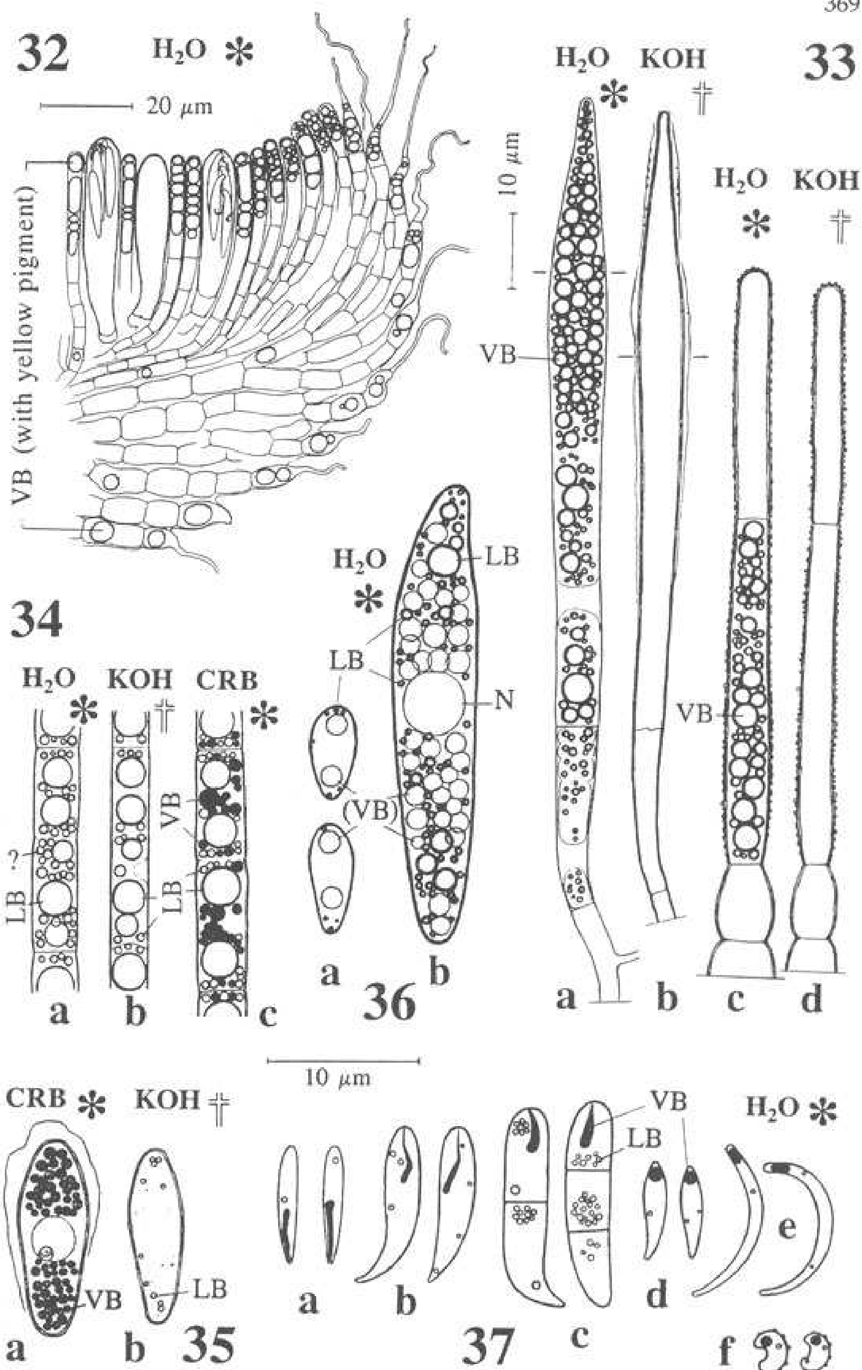
6.c. KOH-soluble cytoplasmic bodies (SCBs)

Vegetative cells of Leotiales may contain globose (FIG. 30) or crystalline (FIG. 31) bodies (here termed *SCBs = soluble cytoplasmic bodies*) of low to high refraction which dissolve in KOH but do not stain with CRB in the living state while adjacent vacuoles are deeply stained. SCBs are localized in the cytoplasm outside the vacuoles. With this set of characters, SCBs resemble WBs but differ in being not associated with septal pores. The contrast of the bodies increases upon staining *in statu vivo* with IKI. Their nature remains unclear; literature reports for Ascomycetes have not been found. GUILLERMOND (1941: 206, fig. 144) described similar crystalloid proteinaceous bodies in cells of lower fungi. Globose SCBs are characteristic of paraphyses of **Pyrenopeziza** (FIG. 30) including "**Pirottaea**", crystalline SCBs occur in paraphyses of **Brunnipila** (FIG. 31) and **Trichopeziza**. **Hyaloscypha albohyalina** var. **albohyalina** (or **H. vitreola**?) is distinguished by the presence of refractive globose to elongate SCBs in paraphyses and excipular cells from **H. albohyalina** var. **spiralis** where these bodies are absent.

6.d. Woronin bodies (WBs)

Most families of Pezizales are characterized by refractive WBs in the cytoplasm a short distance from the septal pores within the vegetative cells, about 3 to 12 per septum, e.g. two on each side (FIGs. 38-41). Their size is about 0.3-0.8(2.5) µm, their shape either globose or crystalline, and their composition proteinaceous

FIG. 32. Section through the margin of **Calycellina ulmariae** (from BARAL, 1989b). Note that the deep yellow VBs occur preferably at the surface of the apothecium. 600x.
FIG. 33. Paraphysis and hair of **Lachnum controversum**. **a.**, **c.** living state, **b.**, **d.** the same cells a few seconds after killing by KOH. KOH dissolves the VBs instantaneously. Note shrinkage of cells especially in width. The horizontal lines on both sides of the widest part of the paraphysis mark the level of the apex of the mature asci. 1500x.
FIGs. 34-37. VBs in ascospores. Note that LBs and VBs in these examples (except FIG. 36) have the same refraction and color, and partly even the same shape. Yet, CRB stains VBs blue but LBs not (FIG. 34c), and KOH dissolves the VBs instantaneously while the LBs persist (FIG. 34b, 35b). Note shrinkage of spores in KOH. **FIG. 34. Tubeufia paludosa. FIG. 35. Hymenoscyphus** cf. **sazavae** (= **Helotium sulphuratum** ss. BARAL & KRIEGLSTEINER 1985: 137). **FIG. 36 a. Cistella deflexa**, **b. Phaeohelotium geogenum. FIG. 37 a. Orbilia sarraziniana**, **b. Orbilia** spec., **c. O. septispora**, **d. O.** cf. **rosella**, **e. O. auricolor** (= **O. curvatispora**), **f. O. delicatula** (= **O. xanthostigma** s.auct.). 2000x. (FIG. 37c from BARAL, 1989a). FIGs. 34 and 35 show the same spore in water (a), and after KOH has been added (b).



(KIMBROUGH, 1991: 425). I have also seen WBs in species of the Sclerotiniaceae. WBs were thought to function as a pore plugging mechanism, sealing off living from dead cells (KIMBROUGH & CURRY, 1986) but KIMBROUGH (1991: 425) now considers non-proteinaceous structures to be more important in septal plugging. Like SCBs, WBs dissolve in 1-5% KOH (but not in nitric acid) and are not stained with CRB. They have often been observed with the LM (e.g. by CORNER, 1929: 271), but can only be seen in living cells.

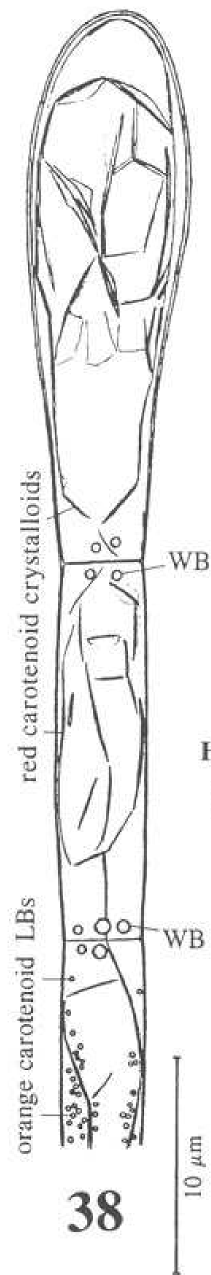
WBs have been used in the systematics of Ascomycetes (e.g. by KIMBROUGH & CURRY, 1986). A special type of WB characterizes **Morchella, Verpa, Disciotis, Gyromitra** (Morchellaceae), and **Discina** (Helvellaceae) (FIGs. 40, 41) but is absent in **Helvella:** several very thin flat crystals of a regular hexagonal outline measuring about 1-2.5/0.2-0.3 µm lie close to the pore. KIMBROUGH (1990; 1991: 422) misleadingly described the WBs of this type as "extremely elongate, rectangular" because, in TEM-sections oriented vertically to the septum, they appear in cross-section as rod-shaped structures. Thicker crystalline hexagonal WBs, however, have been found with the TEM in several non-morchellaceous genera (FIG. 39b; KIMBROUGH 1991: 425).

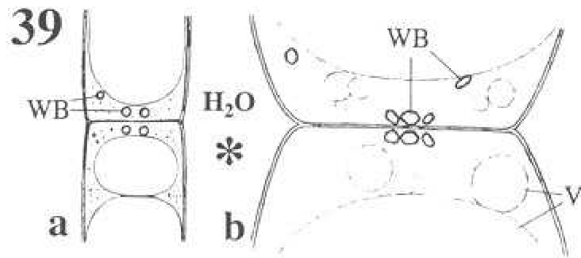
6.c. Pigments

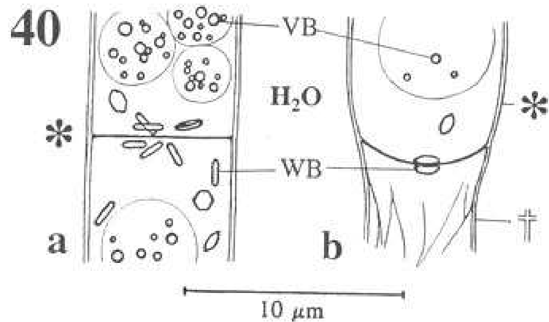
The color of living hymenia may originate from *ectochroic* (= extracellular), *mesochroic* (= cell wall), or *endochroic* (= intracellular) pigments. The first two appear to be typical for long-living discomycetes and are usually unaltered in herbarium specimens. Note, however, that taxonomic problems arise in regard to spore wall pigmentation (see chapter 8). The endochroic pigments are more or less state-dependent and therefore here of special interest: the hymenial color may originate from *cystochroic* (water-soluble, within vacuoles) and/or from *lipochroic* pigments (lipid-soluble, within LBs).

1. Water-soluble pigments (yellow, orange, greenish, bluish or brownish) occur within the VBs of the intact vacuoles of the paraphyses. These pigments may disappear instantaneously (LM, tap water) when cells die during examination in water mounts. On the other hand, a color change of hyaline VBs to yellow, reddish or brownish is often observed and results in deeply colored apothecia which have originally been white. Therefore, HUHTINEN (1990a: 71) describes as *yellow pigment* (often in the dead state) what I differentiate into *hyaline VBs versus yellow VBs* (living state). He consequently merges **Calycellina lachnobrachya** and **C. araneocincta** which have both more or less yellow VBs in the dead state, but differ in the living state (hyaline versus deep yellow VBs; BARAL, 1989b), and occurrence on different hosts. HUHTINEN (l.c.) found, however, that **Hamatocanthoscypha uncipila** is characterized by a yellowish pigment which is *seen clearly only when fresh material is studied in water.*

2. Lipid-soluble yellow to red pigments (carotenoids) occur within globose LBs. These are located in the cytoplasm between the vacuoles, especially in the







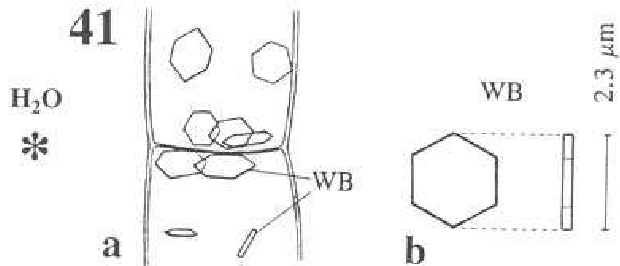


FIG. 38. Pigments and WBs in paraphysis of **Scutellinia scutellata**. Carotenoids form elongate red crystalloids towards the apex but occur also within minute orange LBs towards the base. Globose hyaline WBs occur close to the septa. 3000x.

FIGs. 39-41. WBs close to septa of vegetative cells. **FIG. 39. Peziza** spec., **a**. paraphysis, WBs globose, **b**. medullary hyphae, WBs crystalline. **FIG. 40. Discina ancilis** (= **D. perlata**), **a**. paraphysis, WBs form thin hexagonal crystals, **b**. the pore is plugged by the WBs due to the death of the lower cell. **FIG. 41. Verpa digitaliformis**, medullary hypha, WBs forming very large thin hexagonal crystals (not all of the hyphae contain such large WBs). **a**. 3000x, **b**. 6000x.

paraphyses and in the subhymenium. Yet, in some genera the carotenoids form elongate crystalloid structures, possibly due to a high concentration (FIG. 38; HEIM, 1947; ARPIN, 1968: 430). These crystalloids are inert to KOH. Carotenoids tend to completely lose color in dried specimens (ERB, 1972: 10); consequently, the crystalloids disappear while the LBs are stable except for their pigmentation. Presence or absence of carotenoids in fresh specimens of Pezizales is considered a character of high significance (ERB, l.c.). In my experience, **Scutellinia** (FIG. 38) can be distinguished from **Cheilymenia** in most species by the presence of carotenoid crystalloids in the uppermost 2-4 cells of the paraphyses (LBs which contain carotenoids occur in both genera).

In the living fungal cell, the cytoplasm outside vacuoles, is generally (? always) without pigmentation, except for LBs and carotenoid crystals. Yet, in dead cells of species with pigmented exudates one often observes a coloration of the cytoplasm (reddish, olivaceous, bluish, brown, yellow etc.) similar to the exudates. Obviously, a colorless precursor molecule occurs in the living cytoplasm of these species which becomes colored either outside the cell wall by active exudation, or inside the cells as they die. This situation leads to differing reports of cytoplasmic color depending upon the living versus dead state of the cells.

6.f. Nuclei (N)

Nuclei can often be discerned in living unstained cells by their nucleoli (NO) and nuclear membrane (FIGs. 1a, 4, 12-18, 21-26, 35, 42-43) allowing a rapid evaluation of cell nucleation. For example, species of Sclerotiniaceae differ in the number of nuclei per ascospore. Staining *in statu vivo* with IKI usually strongly enhances contrast of the nuclei (CORNER, 1929: 264) whilst CRB_A, applied to living cells, often stains the nucleolus pale blue. Nuclei are indiscernible in dead, unstained cells and shrink considerably as they die (FIG. 12 a→b). CB often stains them a darker blue than the cytoplasm (FIG. 12b).

6.g. Tracti and "nasse apicale"

A tractus to which the spores are attached occurs in several groups (BARAL, 1987b: fig. 17; 1989b: pl. 3, D), e.g. in **Lecanora** (FIG. 9a, perhaps the first report in this genus). A *nasse apicale* was observed in asci of several species of both fissitunicate and unitunicate Ascomycetes (CHADEFAUD, 1942; BARAL, 1987b: figs. 17, 25), e.g. in **Tubeufia** as a quadripartite structure (FIG. 7). The contrast is enhanced by staining *in statu vivo* with IKI, or it is stained greyish by chlorazol black. Tracti are masked in dead asci by the cytoplasm (LM, FIG. 9b), but have been reported in TEM-investigations. The existence of the *nasse apicale* is doubted, e.g., by REYNOLDS (1971) who interpreted CHADEFAUD's structure as striae on the

inner surface of the endotunica resulting from the banded pattern of the microfibrils. My observations in both Leotiales and Dothideales, however, clearly indicate that the *nasse apicale* is a cytoplasmic structure able to detach from the endotunica.

6.h. De Bary bubbles (DBBs)

Living cells with non-elastic walls compensate for water loss by (FIG. 11 c-h) wrinkling, by collapsing or, in cells with rigid thick walls, by forming DBBs, a gas of presumably water vapour (INGOLD 1956). DBBs are consistently absent in water mounts of living hydrated spores and soon disappear if dry living spores are rehydrated: dry spores of **Hypoxylon serpens**, collected recently and 15 years ago, when placed in water lose DBBs within about 0.5-2 min. Then, DBBs are rapidly induced anew within about 10-15 sec by adding CB, MLZ etc. to the water mount (see also DODGE, 1957). DBBs are also seen by the common practice of mounting fresh or dried ascocarp fragments directly in CB or MLZ. On boiling MLZ slides of **H. serpens** several times, DBBs do not disappear. They disappear only when the MLZ is removed by water. Likewise, HUHTINEN (1983) found DBBs in various genera to be present in MLZ or heated lactophenol but to disappear by gentle heating of a water, Congo red, or KOH mount.

Yet, after having brought an MLZ or a water mount of **H. serpens** to boiling, it proved impossible to induce DBBs anew by adding MLZ (the heated MLZ must be replaced with water until the DBBs have disappeared), which indicates that heating has killed the spores. Collapsing in MLZ occurred in not fully mature spores of this species, including both living and dead spores. This clearly indicates that, in this species, the spore wall is impermeable to the chloral hydrate of MLZ.

DBBs have been introduced as a taxonomic feature by workers who are used to study herbarium material or to mount in CB, MLZ etc. Those, however, who usually mount fresh ascocarps in water wonder why they never see DBBs. From the above, the formation of DBBs within some seconds by adding MLZ or the like to a water mount can serve as a test for vitality.

7. ALTERATIONS IN THE CHEMICAL REACTIONS

7.a. KOH-reaction of VBs

A sulphur-yellow reaction of hyaline VBs to KOH characterizes many mollisiaceous fungi, e.g., **Nimbomollisia eriophori**, **N. melatephroides**, **Mollisia phalaridis**, **Tapesia rosae**, **T. prunicola**, **T. fusca**, **T. hydrophila**, **T. retincola**, **Obtectodiscus aquaticus**. This reaction has been observed in 22 out of the 45 species with VBs so far studied, with some showing variations. SVRČEK's (1986)

description of **Mollisia alcalireagens** is mainly based on the yellow KOH reaction, which he thought to be exceptional in this "new species", and thus needs critical revision. The reaction in **Obtectodiscus** supports the idea that this genus is close to the Dermateaceae as was supposed by SCHEUER (1988: 128).

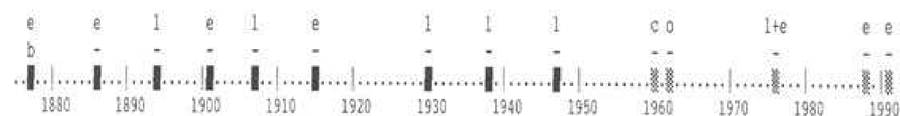
The following method is used: KOH is added to the water mount. The yellow color instantaneously appears around the ascocarp as soon as the KOH reaches the paraphyses, but is rapidly diluted in the medium and soon becomes invisible. The KOH concentration may vary from 2% to 10% while 0.5-1% is much less efficient. The reaction is often absent in senescent ascocarps but was still present in full strength in 0.5 and 4 years old **M. phalaridis** and in 14 years old **T. rosae**. Perhaps, old material fails to react or gives inconsistent results since NANNFELDT (1986: 197) wondered why he could not obtain the reaction in any species. Yet, DENNIS (1950: 182) observed a sulphur-colored KOH reaction in the type material of **M. junciseda** collected in 1868. It is therefore surprising that this reaction has very rarely been reported although KOH is in current use for mounting dried fungi, perhaps because the yellow color had disappeared before the KOH-mount was studied. **Scutomollisia russea** differs in having deep orange VBs which reversibly turn deep violet by KOH.

7.b. Hemiamyloidity

The red (hemiamyloid) reaction of certain wall layers of lichen asci observed in IKI but not in MLZ (BARAL, 1987a; COMMON, 1991: 96) is converted to a uniformly blue reaction not only when KOH-pretreated, but also by preservation in the herbarium for at least about one century (KILIAS, 1981: 256, 410; RAMBOLD, 1989: 37). Conversion from types RR and RB (hemiamyloid) to BB (euamyloid) means that the different types of hemiamyloidity which serve as features of high taxonomic value are irreversibly lost in old herbarium specimens. This conversion is not related to the living versus dead state of the asci. It can now also be reported for the apical rings of the Leotiales:

Tests were made in the summer of 1991 on the genera **Pezicula** and **Ocellaria** (Leotiales), which are defined by apical rings reacting hemiamyloid (type RR). Three species of **Pezicula** and one of **Ocellaria** were studied. 15 collections made between 1877 and 1991 were examined, 11 of which (1877-1963) were received from the Staatsherbarium Munich (M): IKI without KOH-pretreatment gave a red reaction (type RR) in 0-31 years old material, while 44-114 years old material consistently showed a blue reaction which only sometimes turned grey to reddish-grey at a high IKI-concentration. The material in M was repeatedly treated in a freezer (-20°C for 2 h) since about 15 years and poisoned prior to this date (D. TRIEBEL, pers. comm.). This valuable generic feature of **Pezicula** therefore disappears in herbarium specimens much earlier than observations on lichens indicate. The MLZ-reaction without pretreatment was, however, still negative in all collections except for the one from 1877 which reacted MLZ+ pale blue. This situation

(blue in 1% IKI but MLZ-) corresponds to that obtained by a weak KOH-pretreatment (BARAL, 1987a: 421, tab. 4) and offers a certain opportunity to recognize hemiamyloidity in old material.



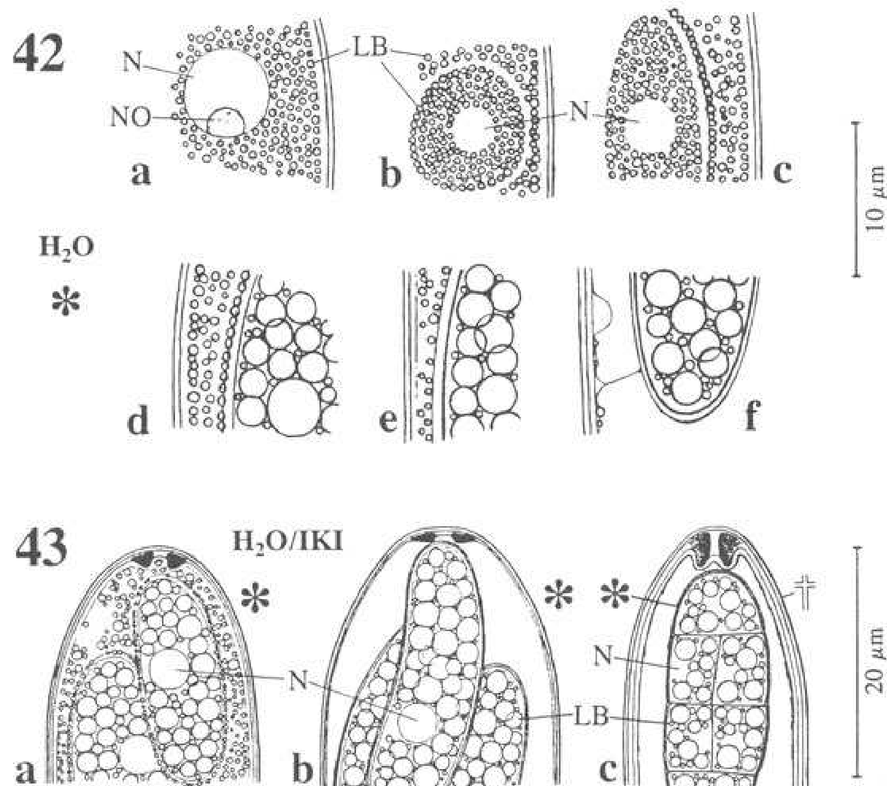
TAB. 2. Iodine reaction (without KOH-pretreatment) of apical rings in 15 collections of **Peziculoideae,** depending on the age of the herbarium material. ▒ = IKI-red, ▌ = IKI-blue. 1st line: l = **P. livida** (asci 8-spored), e = **P. eucrita** (here understood as a 4-spored species), c = **P. cinnamomea**, o = **Ocellaria ocellata**; 2nd line: b = MLZ-pale blue, - = MLZ-negative.

8. ALTERATIONS DEPENDING UPON THE DEVELOPMENT STAGE OF THE CELLS

The development stage of fungal cells can be determined more precisely in the living state. This is of special importance with ascospore characters: number of septa, wall pigmentation, ornamentation, size, and lipid content often markedly differ when comparing the stages of immaturity, maturity, first and second phase of germination. The first phase of germination (GARRAWAY & EVANS, 1984: 221) differs from the stage of maturity by morphological changes, and may either lead directly to the protrusion of a germ tube (second phase of germination), or may persist for a long period of time (dormancy; such spores often show a light brown wall pigmentation though having been hyaline at maturity).

In vital taxonomy, maturity of ascospores can be understood in a very narrow sense: I term ascospores *mature* when actively discharged by the internal pressure of the living ascus, either in a humid atmosphere, or in a water mount during microscopic examination, without applying external pressure. A few hours after natural discharge, however, the spores may already have passed into the first phase of germination, provided that the water mount was protected from evaporation.

BOUDIER (1885: 94) emphasized the fact that spores are often less variable than usually believed when examined in the mature state, i.e. *sorties naturellement de la thèque*. Spore diagnoses should therefore consequently be based on mature spores which have been naturally liberated by explosion of the asci in water mounts. Spore characters in the phases of germination should be evaluated separately and kept apart from those of the mature spore as was done, e.g., by WOLLENWEBER (1939).



FIGs. 42-43. Ascosporogenesis in **Pezicula**. Note changes in spore width and septation (FIG. 43 b→c), and in lipid content. Living asci are required in order to determine the development stage of spores. Septate spores never occur inside living asci of **Pezicula**. **FIG. 42. P. cinnamomea, a.** fusion nucleus, **b.-e.** spore formation, **f.** mature spore; **FIG. 43. P. livida, a.** immature spore, **b.** mature spore, **c.** spore in the first phase of germination. FIG. 43 ab from BARAL (1987b). 2000x.

Ascospores are often 1-celled when mature but 2- to multi-celled in the first phase of germination. Thus, in certain species, a collection of young mature apothecia shows consistently 1-celled spores both within and outside asci while a collection of senescent apothecia shows predominantly multi-celled spores. Other taxa, however, show 2- or multi-celled spores already in the stage of maturity. It is therefore unwise to consider spore septation a variable feature with little reliability, as is often done. Usually, there is little variability if we carefully separate the different

development stages of ascospores. This is only possible if we study living asci and ascospores.

The species of **Pezicula** discharge their spores consistently in the 1-celled stage while muriform spores occur only in the germination phase (FIG. 43). Likewise, **Velutarina rufoolivacea** discharges consistently hyaline spores. These may later become brown (delayed pigmentation), either outside asci or within dead asci but never within living asci. Other taxa, however, have septate, or brown spores inside mature living asci.

A further example is the phenomenon of ascosporal phialidic conidia born directly on ascospores: The term *ascoconidia* has often been misapplied to conidia produced inside dead asci by the budding of ascospores; it should be restricted to conidia which are produced from ascospores in a premature stage of development of the ascus (true secondary polyspory). Ascoconidia are actively discharged in aggregations of 4-8 "balls" which at first glance simulate mature ascospores (FIG. 10a). BREFELD (1891: 293, pl. XI, fig. 41.3) described these balls in **Tympanis truncatula**. Species of **Claussenomyces** can easily be divided into two larger groups, one with true ascoconidia (e.g., **C. olivaceus**), the other with conidia produced only on ascospores outside asci or within dead asci but never within living asci (e.g., **C. prasinulus**). Likewise, **Tympanis**, **Rhamphoria** and **Nectria coryli** produce ascoconidia, while, e.g., **Ascocoryne** and **Pezicula** produce conidia only on ascospores following natural discharge or within dead asci, and are therefore not polysporous. MARTENS (1936: 385) wrongly considered the asci of **C. prasinulus** and **Ascocoryne cylichnium** as secondarily polysporous. Likewise, HAFELLNER & BELLEMÈRE (1983) were unable to distinguish between true secondary polyspory and conidia produced on naturally discharged ascospores. They reported conidial formation within (dead) asci of **Brigantiaea**, leaving the question open whether or not the spores are ejected prior to budding.

Since fresh ascocarps mostly contain both living and dead asci, and since it is impossible to see from the dead specimen which asci have been alive before desiccation, one is completely unable with herbarium material to decide whether true ascoconidia were present, or whether the spores were hyaline or brown, 1-celled or septate in the mature living ascus. Thus, taxonomists working only with herbarium material are often not prepared to refer such a "variation" to certain development stages of the spores. HAFELLNER & BELLEMÈRE (1983: 175) and HUHTINEN (1990a: 70) have stressed this problem: the authors were only able to consider intuitively "good-looking" spores as "mature", and to ignore "bad-looking" spores as "very old" or "senescent". This subjective method, however, leads, in my experience, to unreliable results.

When drying discomycetes for the herbarium, we should be aware that the dry air often induces most of the mature asci to shoot their mature spores, which represent the standard condition for spore descriptions, rapidly into the air. Herbarium specimens then exhibit predominantly immature spores within the asci, or discharged spores in different phases of germination lying on the hymenium, although the apothecia showed full maturity when collected.

The precise development stage can be recognized by the appearance of the living cytoplasm of spores and asci: immature asci show many small vacuoles and hydrated cytoplasm (FIGs. 15ab, 16ab; CHADEFAUD, 1938), which may contain many small LBs in the *pars sporifera* (FIGs. 42 a-e, 43a); CRB induces the formation of MCs in these vacuoles (FIG. 4). The cytoplasm of the spores shows an increase in number and/or size of LBs during sporogenesis (FIGs. 15, 16, 42 c→d; see also JOHNSON, 1963). Mature asci show strongly dehydrated cytoplasm and a high percentage of "free space" represented by one large vacuole which is filled with transparent water (FIGs. 8a, 9a, 10a, 19, 20, 43b); CRB gives a homogeneous violet stain to the large vacuole, while no MCs are formed; the *pars sporifera* reaches its minimum length.

9. DROUGHT TOLERANCE (= POIKILOHYDRY)

When herbarium material collected within the last few years is studied in water mounts, living cells may still be met along with dead cells in a quantity of species. Spores, especially, are known to survive in some cases for many years (SUSSMAN, 1968). Drought tolerance of fruit-bodies is well-known in a few Agaricales and in many Polyporales and Tremellales (INGOLD, 1986: 578).

Fungal cells usually lose a considerable amount of water when passing into the dry, dormant (still living) state. We must therefore distinguish between *in statu vivo* (living state) versus *in statu emortuo* (dead state) on the one hand, and *in statu umido/udo* (hydrated or moist state) versus *in statu sicco* (dry state) on the other hand. Only few fungi perform a strategy of drought avoidance (e.g. **Daldinia**; INGOLD, 1954: 101). In most pyrenomycetes I found little or no structural check on evaporation: the hymenium desiccates and recovers repeatedly, and the perithecial cavities are completely filled with air in the dried dormant state.

Since vitality of single ascocarp cells can easily be recognized under the LM, I carried out tests on tolerance to dehydration: asci and paraphyses of some selected species of Leotiales and Pezizales did not survive even a few minutes or hours in the dry-air conditions of the herbarium while others survived several months. About 50% of either asci or paraphyses were found to be viable after preservation *in statu sicco* at 18-25°C and about 60% relative humidity for the following period of time: (paraphyses {P} were often more tolerant than immature asci, and these more than mature asci {A})

<1 day: **Ciboria caucus** {AP}, **Peziza succosa** {AP}, **Hymenoscyphus fructigenus** {AP}, **H. rhodoleucus** {AP}, **Ombrophila violacea** {AP}

1-2 days: **Lachnum pudicellum** {A}, **L. subvirgineum** {AP}, **Dasyscyphella nivea** {A}, **Sarcoscypha coccinea** {P}

1-2 weeks: **Trichopeziza mollissima** {AP}, **Brunnipila clandestina** {AP}, **Dasyscyphella crystallina** {P}

3-5 months: **Encoelia furfuracea** {AP}, **Lachnellula occidentalis** {P}, **Capitotricha rubi** {P}
8 months: **Lecanora conizaeoides** {AP}

INGOLD (1954: 97) erroneously assumed that fungal spores are "normally without vacuoles" and therefore drought-tolerant. From the above we must conclude, however, that even cells with large vacuoles (asci, paraphyses) are able to withstand strong and prolonged desiccation. Small vacuoles are often present in mature spores (FIGs. 34-37). From the results on paraphyses, I conclude that even wall thickness is unimportant for a cell to be drought-tolerant.

Fungal cells, including mature spores, mostly have very high water contents (60-90%; READ et al., 1982: 2072) in the state of full vitality and hydration (if ample external water is available). Measurements of low total water contents in spores (YARWOOD, 1950; SUSSMAN, 1968; 5-25% for conidia and ascospores) refer to spores which have been in equilibrium with the dry laboratory environment (*in statu sicco*, 22-24°C, 42-51% relative humidity). The spores have *probably lost a considerable portion of their water in the process of collection* (YARWOOD, l.c., often by collapsing), having thereby passed into a state of dormancy. *Naturally collapsed, viable fungal spores are probably of common occurrence in nature* (BECKETT et al., 1984: 94), owing to their inability to check evaporation. *On wetting, they imbibe water and rapidly swell* by regaining their original size (BECKETT et al., 1984: 87). This phenomenon of low water content is called *anhydrobiosis*. SUSSMAN (1966: 740) believed that anhydrobiosis plays no major role in the dormancy of fungal spores, probably because he thought the wall of the mature spore to be quite impermeable to water.

10. CONCLUSIONS

Studying dead cells from fungi preserved in herbaria has very often led to erroneous taxonomic conclusions. It means disregarding many features of high taxonomic importance which have become obscure or have completely disappeared during the death of the cells. Being unaware of the numerous method-induced alterations presented in this paper means working with incompatible observations made on different states or development stages of the cells. Many taxa, even on generic level (e.g. **Mollisia** s.l. versus **Pyrenopeziza** s.l.), are easily distinguished with fresh material but can often hardly be recognized in the dead state.

Many published theories on ascus function and the mechanism of spore discharge which were based on the study of dead asci turned out to be in part erroneous, while DE BARY's (1887) observations on living asci have still high importance and validity. TEM-investigations should routinely be accompanied by LM-studies of the living cell in order to be aware of the changes affecting both cell wall and cytoplasm.

If taxa concepts have been worked out on the basis of abundant fresh collections, as I have done, e.g., in the "**Sarcoscypha coccinea**-complex" (BARAL 1984), one is more or less able to recognize these taxa also from dried material on the basis of correlated features being unaffected by the death of the fruit-body. Yet the process of getting new ideas and arriving at new taxonomic concepts heavily depends upon the applied method: the species of **Sarcoscypha** clearly differ in the size of the lipid bodies inside the living mature ascospores (FIGs. 23-26, the numerous minute accessory LBs occur in all 4 species). This is the most conspicuous feature in that group (apart from striking ecological differences) which independently led BOUDIER and me to the idea that different taxa are involved. In a part of the dead spores of all these species, however, the lipid forms large variable aggregations of comparable size, so this distinction is strongly obscured. HARRINGTON (1990: 436) confirmed this experience: *although I had examined material (dried herbarium specimens) from western North America I was not prepared to recognize that group as a species distinct from the two, large eastern North American species until I saw fresh (living) material*. LE GAL (1941) was unable to distinguish between **S. jurana** and **S. coccinea** in European material because she studied only herbarium specimens.

Those who are not skilled in recognizing vitality of single cells may even be unimpressed when examining cell contents of fresh specimens because, very often, living and dead cells can be found in a single preparation. Thus, one observes a broad scope of morphological and cytological "variation". BOUDIER (1886: 143) stressed this *relative variability* concerning lipid bodies in spores, a variability which is only manifest if one does not carefully separate living from dead cells, and mature cells from those in other development stages.

I have now studied about 9000 collections of Ascomycetes in the fresh, living state (nearly all of them were admittedly collected in Central Europe), and a further 900 in the dried, mainly dead state. Personal communications revealed, however, that mycologists usually consider that, for various reasons, they are not always able to study a major portion of their specimens in the living state. *A monographer receives material from all over the world, most of which is in the dead dried state. It is mainly his personal collecting effort that will enable him to study fresh material* (S. HUHTINEN, in litt.). Some monographers have not even macroscopically seen some or most of their treated species in the fresh state. The method of studying fungi preserved in herbaria seems fairly convenient and highly advantageous at first sight due to the possibility of comparing critical taxa simultaneously. The present study, however, presents a lot of arguments for a precise taxonomy based on the study of living cells. The results obtained by this method are considered so superior and the conflicts resulting from ignorance of the facts presented here are so important, that it is urgent for everybody to reflect on the methods practiced so far. Even describing "clear" new species which are thought to be easily recognizable from persistent characters is of limited taxonomic value since critical unknown taxa close to every such "clear" taxon may be discovered in the near future.

Vital taxonomy means to be ready for study whenever a species is collected or is received from a colleague by post. Ascomycetes, or even Basidiomycetes (KOIVURINTA, 1978), can be stored in the refrigerator at about +5-10°C for several days or even weeks without any ill effects. The great value of vital taxonomy is that a relatively large amount of microscopic data can be gathered in a reasonable period of time and with higher efficiency concerning the value of the results. SVRČEK (1976: 116) wrote: *In fact, the study of dried specimens as such is much more difficult and more time-consuming than work with fresh material.* Furthermore, vital taxonomy means frequent and regular field work. Herbarium taxonomy has resulted in a deficiency in our knowledge of ecological preferences of a species. Many species are known only from the type collection, the host on which they grew being often unknown, and many species are said to be difficult to find. The experience of G. MARSON (pers. comm.) and me is, however, that many species are of more common occurrence and are consequently available for a study *in statu vivo* at a much higher frequency than is usually believed.

A problem arises in the typification of taxa being mainly distinguished by transitory characters (which are only visible in the living state). According to Art. 9.1, ICBN (GREUTER et al., 1988), the type of a species must be a dried specimen or microscope slide). Living plants or cultures are not permitted (Art. 9.5, ICBN). Illustrations rank second and are only accepted as types if specimens are lacking or cannot be preserved as dried specimens (Art. 9.3, ICBN). This method requires the recognition of the species from the dead state. Should we therefore consider characters which are visible only in the living fungus to be less valuable for taxonomic purposes?

According to BRESINSKY (1964), exsiccata especially in Agaricales, exhibit much less distinctive macroscopical differences when compared with the living basidiocarp. In order to emphasize the value of microscopic features in this group and to facilitate the study of type material, he produced keys to exsiccata which either allow the determination of species, or only taxa of somewhat higher level. I suppose, however, that these keys are predominantly based on pre-existing taxa concepts which had been prepared on the basis of macroscopical characters of fresh, living basidiocarps. Thus, ORTON (1960: 161) recommended to study fresh Agaricales and Boletales both *macro- and microscopically, before pronouncing a verdict on the dried material.* Concerning the Pezizales, BENEDIX (1972: 163) stated that dried material alone is insufficient for taxonomic decisions on critical, mainly macroscopically defined species.

Nevertheless, monographers have probably very often prepared their taxa concepts on the basis of dried material. In the higher plants, for example, LEENHOUTS (1968: 26ff.) recommended to find out taxonomical entities by carefully comparing a large number of herbarium specimens ("herbarium taxonomy").

We have to acknowledge that dried specimens do not reflect either the macro- or the micromorphology of the living state. Since there exist many state-dependent features, it is necessary to describe these features from living cells in water and note anomalies caused by the death of the cells or by reactions to mountants (this was also recommended by SPAIN, 1990: 75). No satisfactory method for the fixation of transitory characters for permanent preparations is known. According to READ et al. (1982), *all preparative procedures that all necessitate the elimination of constituent water used in SEM produced artifacts.* It is therefore highly desirable that drawings, photographs, and descriptions of living fungal organs are deposited together with the dried specimens. Microscopic measurements should mainly be taken from living cells in water (although these data are incompatible with those obtained from herbarium material) since the stage of development of the organs cannot clearly be recognized after mounting in lethal media (Note that HUHTINEN, 1990, gave measurements of spores and asci, whenever possible, *when fresh in water* along with those in MLZ or CB).

In order to prepare more precise taxonomic concepts, both extended field work and immediate microscopic study is necessary. Monographers are urged to make as many personal fresh collections as possible. Due to the substantial loss of valuable vital characters in dead fungi, descriptions of type material *in statu vivo* have high importance. If an Ascomycete taxon cannot satisfactorily be recognized from dried specimens, the protologue, if based on living specimens, must automatically rank first in the typification of its basionym. New taxa should therefore be described from living specimens whenever possible.

11. HOW TO MAKE VITAL TAXONOMY

11.a. Method

1. How to collect:
- drought-intolerant Ascomycetes:
  - use boxes of watertight material (no paper boxes)
  - produce a humid atmosphere inside by adding fresh moss etc.
  - avoid mechanical pressure
- drought-tolerant Ascomycetes:
  - can be collected in dry or fresh condition
  - rehydrate dry fruit-bodies a few minutes prior to preparation by spraying with water

2. How to make preparations for the LM:
  - mount in tap water
  - avoid long-time exposure of ascocarps to dry air or warm light
  - place the fungal fragment immediately into the water drop on the microscope slide in order to avoid critical desiccation
  - cut freehand through the hydrated ascocarp under the dissecting microscope
  - stain *in statu vivo* by adding CRB or IKI to the edge of the coverglass

- add toxic (lethal) reagents to the edge of the cover glass to observe alterations
- note whether each described cell was alive or dead
- employ (osmotically inert) viscous solutions of albumin for photomicrography in order to prevent movement of spores
- keep immature collections for some days in the box to obtain mature hymenia
- allow some apothecia to deteriorate in order to study ascospore germination and possible production of conidia on the ascospores

11.b. Important vital characters

1. Asci:
   - measure living, mature asci first because these readily liberate the spores in many genera
   - observe presence or absence of croziers which can readily be seen in sections of living young apothecia (apply no pressure on the coverglass!)
   - study the apical apparatus prior to discharge in both the living and the dead state; employ IKI for diagnosing blue versus red reactions

2. Spores:
   - take measurements and observe spore characters from living mature spores recently shot into the medium by active spore discharge (spores inside living asci are often distinctly narrower though mature due to the ascus turgor, and spore characters like pigmentation, septation etc. strongly depend upon the development stage)

3. Sterile tissue:
   - study excipular structures from living sections which are not too thin (approx. 30-100 μm)
   - recognize imbibition of water by intercellular gel in the dead state when applying CB or MLZ to the section (textura oblita in the dead state may look like textura prismatica in the living state!)
   - observe cell contents in living paraphyses, hairs, and excipular cells, especially properties of the VBs: refraction, shape, size, color, location

11.c. How to study dead herbarium specimens

- use water first in order to prohibit dissolution of wall deposites or exudates, and to test the IKI reaction for hemiamyloidity
- use 2% KOH for the observation of the lipid in the cells, especially in the spores (the lipid in dead cells is often masked in nearly all other mountants in use)
- the ascogenous hyphae can be studied for croziers by mounting in KOH or in Congo red (strong pressure on the coverglass and heating may be necessary)

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