Studies of the Vegetative Mycelium in the Genus Agaricus L.: Fr. emend. Karst.

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In this paper on microscopic and other studies on the vegetative mycelium of 17 species of the genus Agaricus authors have report a number of new characteristics which seem to have taxonomic significance for certain species within this genus. These are in particular: presence of clamp connections, asexual sporulation, relative DNA content and number of nuclei per cell, the last property possibly having also some ecological significance. Fluorescence of the vegetative mycelium was characteristic for all Agaricus species investigated. Most of the properties of vegetative mycelium of the reported Agaricus species were unknown so far. Authors conclude that using micromorphological criteria of the vegetative mycelium in addition to cultural properties, it seems to be possible to establish new taxonomical criteria in the genus Agaricus, being typical also for other Agaricales.

Introduction

Cultural and morphological properties of sterile vegetative mycelia of higher Basidiomycetes are often used in the study of taxonomic, developmental and biotechnological problems (Miller, 1971; Nobles, 1971; Stalpers, 1978; Buchalo & Wasser, 1981; Šašek et al., 1986; Buchalo, 1988; Semerdžieva et al., 1988; Klan, Baudisova & Ruflova, 1992; Buchalo et al., 1994a, Molitoris, 1995). The vegetative mycelium in Basidiomycetes, like in most other fungi, represents a complex of differently branched hyphae, which differ only within narrow limits of width, length, number of nuclei, thickness of cell walls and the character of branching. On the basis of statistical evaluation some authors as Parmeter (1965) conclude that the vegetative mycelium in many fungal species is similar and cannot be used as a reliable taxonomic feature. However, continious accumulation of information on an increasing number of fungal species provides now material for study and comparison of morphological characters and for their potential use for taxonomic purposes.

In higher Basidiomycetes a great diversity in hyphal morphology was described and some of the forms observed may have a taxonomic importance. A few suggestions for classification of hyphae on the basis of their physiological role, type of branching, cell wall thickness, presence of aggregates on the surface or inside the cells, etc., were made (Lohwag, 941; Nobles, 1965; Donk, 1971). On the mycelia different types of bristles, spines swellings, bulbs, hyphal tangles, monilial hyphae, gloeocystids are formed, some of which may be exploited for morphological characterization of cultures permitting identification of fungal species (Nobles, 1965; Miller, 1971; Stalpers, 1978; Pantidou, Watling & Gonou, 1983; Buchalo, 1988; Buchalo, Šašek & Zakordonec, 1989; Jacobsson, 1989). Stalpers (1978) e.g. presented a description of 26 types of hyphal modifications, many of them, in our opinion, being hardly distinguishable.

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Morphological characters of vegetative mycelium of Aphyllophorales and of some Agaricales were by several autors investigated and used for the identification of species in the vegetative stage of growth (Nobles, 1958, 1965, 1971; Watling, 1977, 1979; Stalpers, 1978; Kendrick & Watling, 1979; Pantidou, Watling & Gonou, 1983; Buchalo, 1988; Reshetnikov, 1991; Klan & Baudisova, 1992). Vegetative mycelium in the genus Agaricus was also studied using light and fluorescent micriscopy (Garibova & Shalashova, 1973; Garibova & Safraj, 1980; Sonnenberg & Fritsche, 1989). In the meantime, using scanning electron microscopy (SEM) some new features could be established for describing more precisely the taxonomic, ecological and physiological status of cultures.

In the present investigation on morphology of vegetative mycelium in cultures of seventeen species of the genus Agaricus SEM was applied for the first time, except a paper on *A. bisporus* by Whitney & Arnott in 1987. Nuclear numbers in cells of vegetative mycelium had been investigated in only 5 species of the genus Agaricus (Wang & Wu, 1974; Elliott, 1979; Hou & Elliott, 1979; Sonnenberg & Fritsche, 1989). Estimation of the amount of nuclear DNA and determination of fluorescence of hyphae was undertaken in this work for the first time.

Materials and Methods

Seventeen species of the genus Agaricus from the Culture Collection of Higher Basidiomycetes of the M.G. Kholodny Institute of Botany of the National Academy of Sciences of the Ukraine were used (Buchalo & Mitropolskaya, 1990). The system of the genus Agaricus following S. Wasser (1980, 1985) was used throughout this paper.

Subgenus Agaricus

Section Agaricus

Subsection Agaricus

A. bresadolianus Bohus, strain (str.) 104;

A. campestris L.: Fr., str. 144;

A. vaporarius (Vitt.) Mos., str. 293;

Subsection Sanguinolentae (J. Schaeff. et Moell.) S. Wasser

A. squamuliferus (Moell.) Moell., str. 124, 158;

A. sylvaticus Schaeff.: Secr., str. 37;

Section Duploannulatae S. Wasser

A. subfloccosus (J. Lge) Pil., str. 292;

A. bisporus (J. Lge) Imbach, str. 4, 36, 288, 289, 290;

A. bitorquis (Quél.) Sacc., str. 143;

A. bernardiiformis Bohus, str. 156;

Subgenus Flavoagaricus S. Wasser

Section Majores Fr. p. p.

Subsection Flavescentes (J. Schaeff. et Moell.) S. Wasser

A. excellens (Moell.) Moell., str. 145;

A. maskae Pil., str. 157;

A. sylvicolus (Vitt.) Pk, str. 17;
A. macrocarpus (Moell.) Moell., str. 114, 150;
A. abruptibulbus Pk, str. 284;
A. fissuratus (Moell.) Moell., str. 208;
A. arvensis Schaeff.: Fr., str. 14, 15, 285, 286;

Subsection Xanthodermae (Sing.) S. Wasser
A. xanthodermus Gen., str. 27, 294.

The cultures were grown on PDA at 26°C. Maintenance of the cultures, preparation of mycelium and scanning electron microscopy techniques were as described in our previous paper (Buchalo et al., 1983). Light microscopy followed Molitoris (1963). Fluorescent microscopy investigations was performed according to Meixner & Bresinsky (1988). Energy dispersive x-ray microanalysis was undertaken using a Link Systems solid state detector and 860 multichannel analyser. The material was examined at 25 kV in a JEOL 35C scanning electron microscopy and spot analysis of the crystals was undertaken.

Results and Discussion

Vegetative mycelium of the species investigated consists of thin-walled hyphae which are weakly branched. The diameter of the hyphae varies between 2 to 4 μm. Anastomoses are formed between hyphae in all species and strains. In some cases numerous anastomoses are formed (Fig. 1). Some other morphological structures were investigated in the vegetative mycelium of several species, such as thickness of cell walls, apical and lateral hyphae. In our opinion the above mentioned structures and anastomoses have no taxonomical significance. Strand-like mycelial cords were found in *A. arvensis, A. bisporus, A. bitorquis, A. campestris* (Fig. 2), *A. subfloccosus, A. vaporarius.* These species represent the ecological groups of coprophilic and meadow saprophytes.

Clamp connections are characteristic features of many dikaryotic mycelia of Basidiomycetes (Buchalo et al., 1983). It is widely accepted, however, that they are not common in all species of Agaricus. Singer (1961) reported the presence of clamp connections in the genus Agaricus but he did not mention definite species. Garibova & Shalashova (1973) during their investigations of mycelial morphology of Agaricus species found clamp connections only in *A. campestris, A. subperonatus* (J. Lge) Sing. and paired clamp-like structures in *A. bisporus.* Clamp connections were observed also in *A. arvensis, A. bernardii* Quél. apud Cke et Quél. (Wasser, 1985; Sonnenberg & Fritsche, 1989) and in *A. comtulus* Fr. (Garibova, 1982). Clamp-like features were described in *A. sylvaticus* (Wasser, 1985). The majority of authors noted that clamp connections occured very rarely in vegetative mycelium of Agaricus. Using light and SEM we investigated the clamps connections in *A. arvensis* and *A. campestris* which occured very rarely. Clamp connections in these species have the classical form but without a slit between the clamps and the septum (Fig. 3).

Stages of anamorphs are important characteristics of pure cultures in higher Basidiomycetes (Watling, 1977, 1979; Kendrick & Watling, 1979; Buchalo et al., 1985; Buchalo, 1988; Reshetnikov, 1991). We observed the anamorphic stage in 8 species: *A. abruptibulbus, A. arvensis, A. bernardiiformis, A. bisporus, A. fissuratus, A. macrocarpus, A. maskae, A. squamuliferus.* In these species, excluding *A. bisporus*, we observed the fragmentation of hyphae in arthroconidia. In *A. bisporus* we observed the formation of thickened cells which we consider representing chlamydospores (Fig. 4).

Arthroconidia usually contain 2 nuclei. They appear in pairs or in chains of 3 or more cells, the diameter of the arthroconidia is 3 to 4 μm, the length is up to 15 μm (Fig. 5).

Crystals. Oxalic acid represents one of the main metabolites of the Krebs cycle in living organisms, including higher Basidiomycetes (Shivrina, 1995; Stalpers, 1978; Buchalo, 1988). Presence of crystals of calcium oxalate (COC) on hyphae was reported in the literature, especially for *A. bisporus*, the cultivated mushroom (Molitoris, 1963; Garibova & Safraj, 1980; Buchalo,1988). COC are formed on the hyphae under cultivation in different nutritional media (agar and liquid media, grain, compost etc.) and represent a relatively stable characteristic of the cultures (Eger & Sücker, 1964; Ivanovich, 1965,.Garibova et al., 1982).

Several hypotheses exist about the role of calcium oxalate in or on fungal cells:

a) disposal of accumulated toxic metabolites (Garibova et al., 1982);

b) mechanical barriers against attacks of bacteria, fungi, arthropods (Holdonrieder, 1982; Whitney & Arnott, 1987);

c) definite role in the hydrophobic cell coating (Whitney & Arnott, 1987);

d) storage of carbon for later utilisation under certain conditions (Badaljan, 1993);

e) age-dependent accumulation indicating the readiness of the mycelium for transition into the generative phase of development (Eger & Sücker, 1964);

f) maintenance of C/N balance through elimination of excess carbon in a nitrogen-poor substrates (Akamatsu et al., 1994).

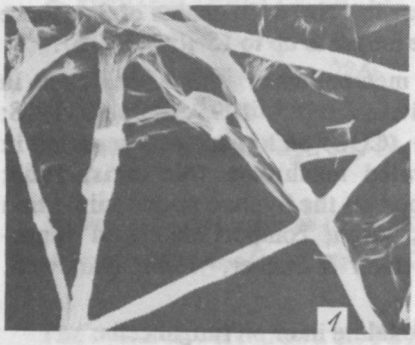
Formation of calcium oxalate can be stimulated by pH regulation or by addition of calcium chloride to the medium (Edwards, 1974).

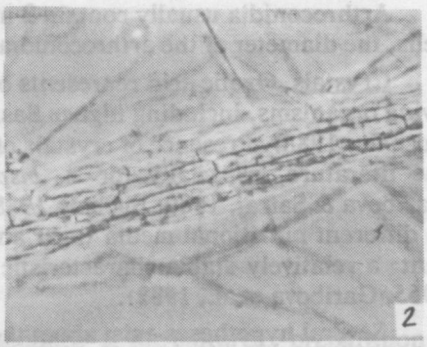
Crystal formation was observed in all species of Agaricus investigated. The density of crystals on the surface of hyphae may vary. Crystals are abundant in the majority of species, *A. abruptibulbus, A. fissuratus, A. sylvicolus,* however, show only a few crystals on hyphae. Different stages of crystal formation could also be observed. Initially, crystalls are formed within the cell wall (Whitney & Arnott, 1987) and finally are located more or less tangentially on its surface (Fig. 6). As a rule, crystals cover the hyphae and are rarely found separated from the cells. Release of crystals from the hyphae possibly could indicate lysis of the cell.

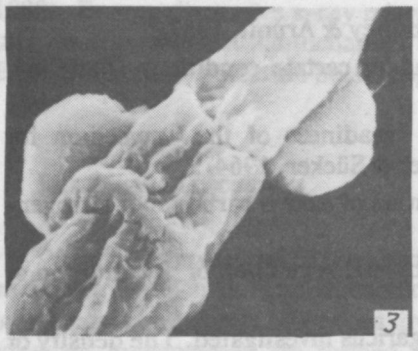
The morphology of the crystals is very different. We observed cubic, hexaedral, pyramidal, bipyramidal, prismatic, rod-shaped and acicular crystals (Fig. 7). Maximal length odservedwas sometimes more than 10 μm, thickness was 1 to 4 μm. Sometimes crystals with undefined shape were observed. Using x-ray microanalysis of single crystals under the scanning electron microscope, calcium could be identified as the cation (Fig. 8). For determination of the nature of the anion the solubility and the concominant gas production of the crystals in various solvents was used: 0.01, 0.1 and 1 N acetic and hydrochloric acids, and 1 N ammonium chloride were used. In the case of acetic acid and ammonium chloride the crystals were not soluble, in hydrochloric acid, however, they dissolved without gas production. This proves that the crystals observed represent calcium oxalate rather than calcium carbonate. Molitoris (1963), Thielke (1966), Whitney & Arnott (1987) have already repoted earlier about the presence of COC in *A. bisporus.*

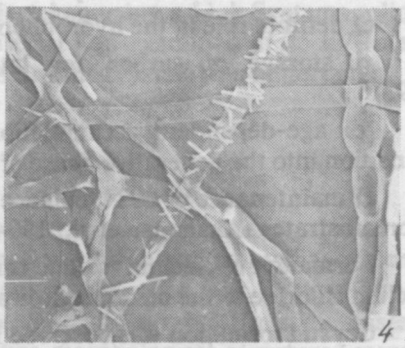
It is important that different strains of *A. arvensis, A. bisporus, A. macrocarpus* etc. show a similar variation in the shape of the crystals observed.

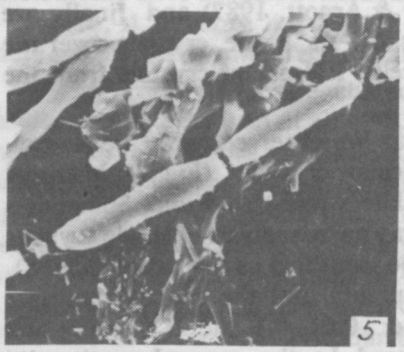
Amoumt of nuclear material (chromosome numbers) had so far little significance in fungal taxonomy because chromosomes of fungi generally are extremely small and are difficult to separate and to count. However, recently it was shown that the DNA content of individual nuclei can be accurately measured with cytophotometry and used in fungal taxonomy (Duran & Gray, 1989; Wittman-Meixner et al., 1989). As an alternative to the chromosome number, or in addition to it, the genome size along with classical

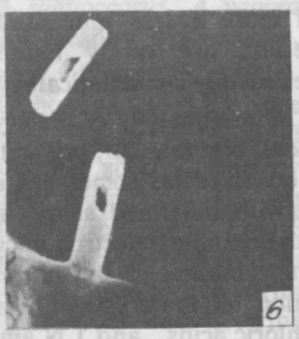


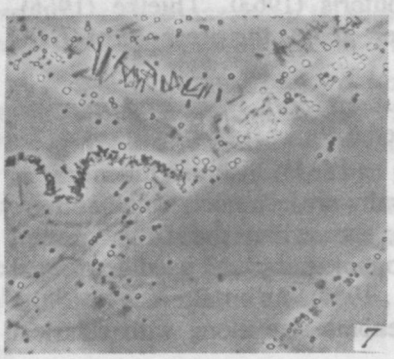












Figures:

1 – Anastomoses in *A. vaporarius* (SEM, x3600);

2 – Mycelial cord in *A. campestris* (LM, x1250);

3 – Clamp connections in *A. campestris* (SEM, x8600)

4 – Chlamydospores and rod-shaped crystals in *A. bisporus* 288 (SEM, x3600)

5 – Arthrospores and crystals of different shape in *A. arvensis* 14 (SEM, x3600)

6 – Crystal formation in *A. campestris* (SEM, x2000)

7 – Crystals of different shape in *A. campestris* (LM, x500).

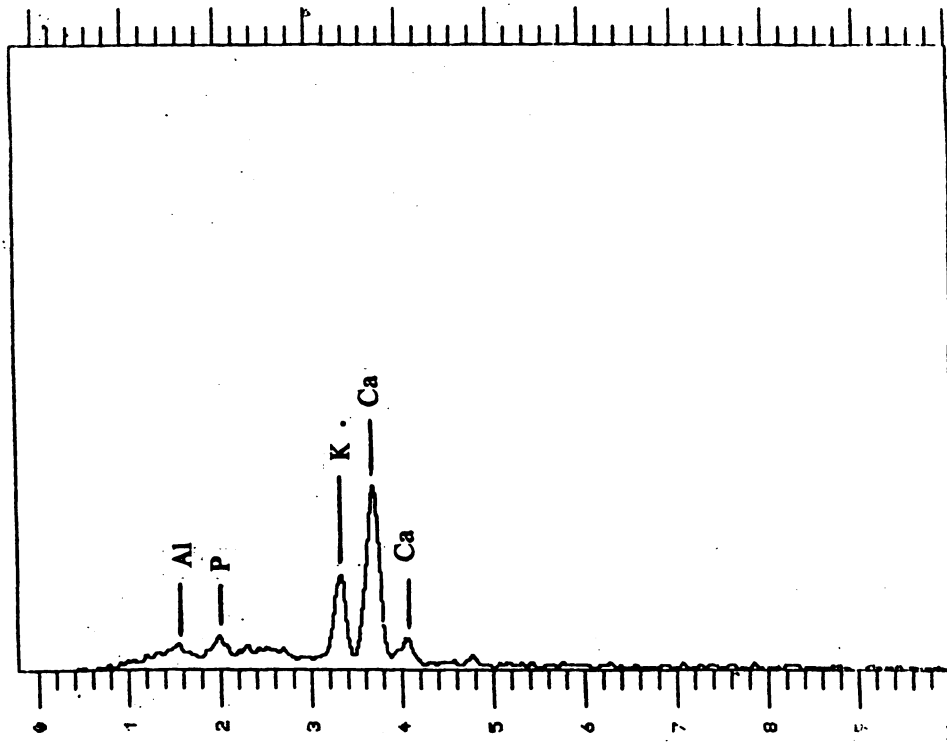


Fig. 8. X-ray microanalysis of a crystal on a hypha of *A. bresadolianus* indicating Ca^{2+} as cation.

morphological criteria, should help the taxonomist to distinguish more critically taxa being similar in morphology, especially if the amounts of nuclear DNA can be shown to differ significantly (Duran & Gray, 1989). The quantification of nuclear DNA is comparatively simple and consistantly yielded reproducible results. Until recently, there have been relatively few quantitative studies of fungal DNA, in particular of DNA of higher Basidiomycetes. Using this method, e.g. different species of Armillaria and Conioforacea were compared and different ploidy levels were found within the genus Pleurotus (Motta, Peabody & Peabody, 1986; Bresinsky et al., 1987; Meixner & Bresinsky, 1988).

In our studies on the genus Agaricus cytophotometric determination of the relative DNA content in nuclei and attempts to find several ploidy levels within this genus using DAPI fluorescent staining were maid.

DNA content of about half of the cultures investigated equaled that of the control (DNA content of the nuclei of *A. bisporus*, str. 4). The lowest DNA content (60-70%), as compared with the control, was registered for *A. abruptibulbus, A. arvensis, A. macrocarpus* str. 114, *A. maskae, A. sylvaticus* (Tabl. 1). The largest DNA content was found in *A. fissuratus* – 220%, *A. macrocarpus* str. 150 – 200%, *A. sylvicolus* – 180%. In the strains investigated within a given species the relative DNA content therefore was similar (Fig. 9, 10). An exeption were only the strains of *A. macrocarpus*. A possible explanation could be polyploidy (*A. macrocarpus* str. 114 = n, *A. macrocarpus* str. 150 = 3n). However, this has to be confirmed by direct observation and count of nuclei in the cells of vegetative mycelium of both strains.

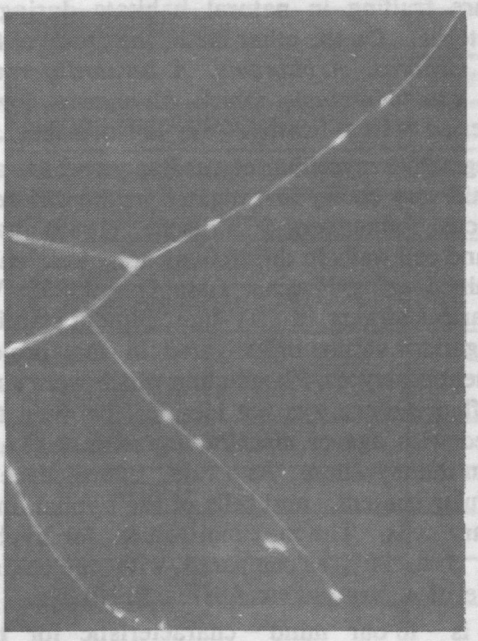
Some data on the correlation of ploidy level with ecology in certain plants and fungi were obtained and discussed by Ehrendorfer (1980) and Bresinsky et al. (1987). The authors showed that DNA content is higher in those species that are exposed to unfavourable habitats (arctic and alpine regions, deserts) and that have a short period of vegetation. Our results suggest a similar correlation in the fungal genus

Tabl. 1. Nuclear DNA amount, length of genome and ploidy levels in investigated species of genus Agaricus

Species	Relative amount of DNA (%)	Level of ploidy
Subgenus Agaricus		
A. sylvaticus	59	n
A. vaporarius	121	2n
A. subfloccosus	118	2n
A. squamuliferus	110	2n
A. campestris	103	n
A. bisporus	95	n
A. bitorquis	101	n
A. bernardiiformis	73	n
A. bresadolianus	156	2n
Subgenus Flavoagaricus		
A. arvensis	65	n
A. abruptibulbus	68	n
A. macrocarpus	64 (196)	n (3n)
A. sylvicolus	184	3n
A. fissuratus	219	3n
A. excellens	106	2n
A. maskae	76	n
A. xanthodermus	95	n

Fig. 9. Amount of nuclear DNA and hyphae fluorescence in species and strains of Agaricus subgenus *Agaricus*.

Fig. 10. Amount of nuclear DNA and hyphae fluorescence in species and strains of Agaricus subgenus *Flavoagaricus*.



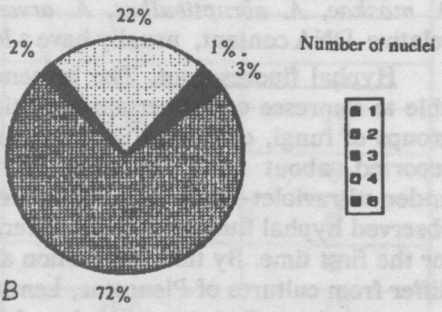
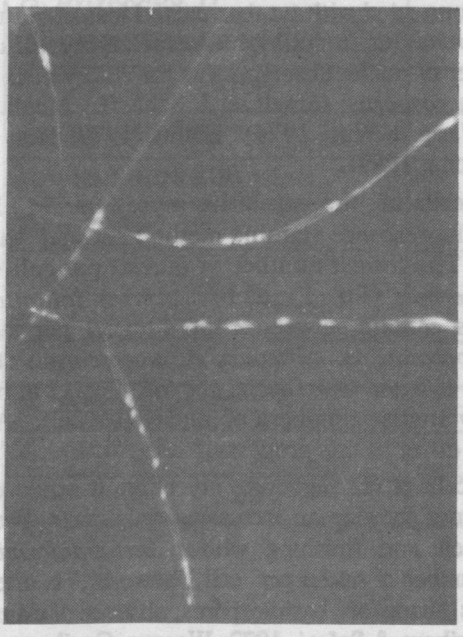


Fig. 11, *a* – Hyphae of *A. xanthodermus* 294 showing compartments with 2 nuclei each (LM, x500)
Fig. 11, *b* – Percentage of number of nuclei per hyphal compartment as calculated from DNA staining with DAPI



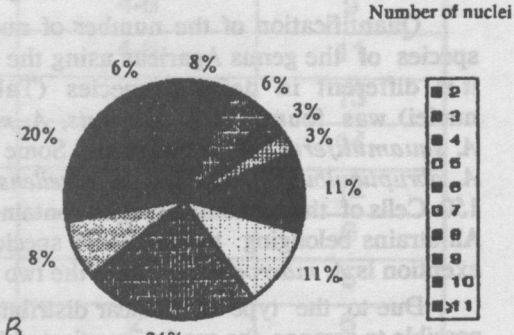


Fig. 12, *a* – Hyphae of *A. sylvaticus* showing compartments with different numbers of nuclei (LM, x500)
Fig. 12, *b* – Percentage of number of nuclei per hyphal compartment as calculated from DNA staining with DAPI

Agaricus. *A. bresadolianus, A. vaporarius, A. squamuliferus, A. sylvaticus, A. subfloccosus, A. excellens, A. sylvicolus, A. macrocarpus, A. fissuratus,* which all show a high relative DNA content (ploidy levels 2n and 3n) are fruiting in natural habitats during a comparatively short period of time (July to October). On the other hand, the group of the remaining Agaricus species (*A. campestris, A. bisporus, A. bitorquis, A. bernardiiformis, A. maskae, A. abruptibulbus, A. arvensis, A. xanthodermus*), which all show a lower relative DNA content, usually have a longer period of fructification (May to November).

Hyphal fluorescence. The presence in vegetative mycelium of substances which are able to fluoresce under certain conditions is still very poorly investigated in the different groups of fungi, especially in the genus Agaricus. Sonnenberg & Fritsche (1989) have reported about the fluorescence of septa and cell walls in different strains *A. arvensis* under ultraviolet light. The data were obtained using Hoechst stain Due 33258. We observed hyphal fluorescence of different Agaricus strains in UV-light without staining for the first time. By this observation all the Agaricus strains investigated in this project differ from cultures of Pleurotus, Lentinus, Kuehneromyces, Flammulina which were used for comparison. Staining intensity of hyphal fluorescence was not identical between the different strains within a given species. It varied with age of mycelium, growth stage and – possibly – with activity of the metabolism in the mycelium. As a rule, fluorescence of the septa was more intensive than of the cellular content, and cells of the hyphal apex showed a higher fluorescence than intercalary cells. The maximal values for hyphal fluorescence varied in different strains from 5 to 20% as compared with the control (intensity of fluorescence of DAPI-stained nuclei of *A. bisporus* str. 4) (Fig. 9, 10).

The phenomenon of hyphae fluorescence is – to our mind – characteristic for the genus Agaricus and after more detailed investigations could possibly be used as an additional taxonomical criterion on the species and genus levels.

Number of nuclei per cell is a very important character of vegetative mycelium in basidiomycetous fungi for the estimation of the taxonomic and evolutionary position of species. On the basis of nuclear characteristics five groups of higher Basidiomycetes can be separated (Boidin, 1971). This author suggests that primitive Basidiomycetes have two nuclei, have clamp connections and are heterothallic. He regards the multinuclear status of higher Basidiomycetes as evolutionary advanced. Kühner (1977) also considers morphological complexity in Basidiomycetes as connected with total or partial loss of clamp connections. The data refer to the nuclear behavior in cells of vegetative mycelium of 5 species of the genus Agaricus. The number of nuclei observed was for *A. arvensis* – 5-7; *A. bitorquis* – 2; *A. bisporus* – 6-8; *A. macrosporus* (Moell. et J. Schaeff.) Moell. – 8; *A. nivescens* (Moell.) Moell. – 8 nuclei (Wang & Wu, 1974; Elliott, 1979; Hou & Elliott, 1979; Thielke, 1985; Sonnenberg & Fritsche, 1989).

Quantification of the number of nuclei in cells of the vegetative mycelium in 17 species of the genus Agaricus using the DAPI fluorescence staining method showed that it is different in definite species (Tabl. 2). The lowest number of nuclei per cell (2 nuclei) was found in *A. bitorquis, A. xanthodermus* (Fig. 11, a, b), *A. bresadolianus, A. squamuliferus, A. subfloccosus.* Some species contain between 2 and 4 nuclei per cell: *A. abruptibulbus, A. maskae, A. excellens, A. vaporarius, A. sylvicolus, A. macrocarpus* str. 150. Cells of the remaining species contain from 4 to 6 and more nuclei per cell (Fig. 12 a, b). All strains belonging to the same species show similar numbers of nuclei per cell. An exeption is *A. macrocarpus* where the two strains differ in this property.

Due to the type of nuclear distribution in cells of the vegetative mycelium it would be possible to arrange the species investigated in an order showing an increasing evolutionary level, beginning with species containing 2 nuclei per cell and finishing with *A. bernardiiformis,* containing 8-10 nuclei per cell. The criterion of number of nuclei per cell, however, is often contradictory to other evolutionary criteria such as shape of basidiospores, absence of clamp connections, level of ploidy etc. (Singer, 1961; Garibova & Safraj, 1972; Wasser, Garibova & Mokeeva, 1976; Wasser, 1980, 1985) A possible explanation for this could be the phenomenon of heterobatmy in the genus Agaricus (Wasser, 1980).

Tabl. 2. Number of nuclei per cell in investigated species

Species, strains	Nuclei per cell		
	Minimal	Overage	Maximal
Subgenus Agaricus			
A. bresadolianus 104	1	2	4
A. campestris 114	3	4-6	12
A. vaporarius 293	1	2-4	8
A. squamuliferus 124	2	2	6
-- 158	1	2	6
A. sylvaticus 37	3	6-8	15
A. subfloccosus 292	1	2	5
A. bisporus 4	2	4-6	8
-- 36	1	4-8	10
-- 288	2	4-6	8
-- 289	2	4-6	12
-- 290	2	3-6	10
A. bitorquis 143	2	2	4
A. bernardiiformis 156	2	4-10	15
Subgenus Flavoagaricus			
A. excellens 145	2	4	8
A. sylvicolus 17	1	2-4	7
A. macrocarpus 114	2	4-6	13
-- 150	1	2-4	6
A. maskae 157	1	2-4	8
A. fissuratus 208	2	4-6	9
A. arvensis 14	2	4-6	11
-- 15	2	4-8	13
-- 285	1	4-8	14
-- 286	2	4-8	12
A. abruptibulbus 284	1	2-4	6
A. xanthodermus 27	1	2	5
-- 294	1	2	6

Conclusion

In this paper on microscopic and other studies on the vegetative mycelium of Agaricus strains we have included some of our earlier observations on cultural behavior in this genus (Bukchalo, 1974; Grigansky & Buchalo, 1994; Buchalo, Šašek & Grigansky, 1994 a, b) and report a number of new characteristics which seem to have taxonomic significance for certain species within the genus Agaricus. There are in particular: presence of clamp connections, asexual sporulation, relative DNA content and number of nuclei per cell, the last property possibly having also some ecological significance. Fluorescence of vegetative mycelium was characteristic for all Agaricus species investigated.

Most of the properties of vegetative mycelium of the reported Agaricus species were unknown so far. By using micromorphological criteria of vegetative mycelium in addition to cultural properties, it seems to be possible to establish new taxonomical criteria in the genus Agaricus, being typical also for other Agaricales. Based on the study of a complete life cycle in higher Basidiomycetes, and not only based on the part of sexual reproduction, as it was usually done, possibly a general species concept for this group of fungi can be established.

ACKNOWLEDGEMENTS

The authors thank S.T. Moss, Portsmouth, for x-ray microanalysis, M. Beisenherz, Regensburg, for the help in fluorescence microscopy and Ch. Thielke, Bovenden, for critically reading the manuscript. One of the authors (A.G.) thanks the Bavarian government for funds for a 5-month research visit to the laboratory of H.P.M. at the University of Regensburg.

ИССЛЕДОВАНИЕ ВЕГЕТАТИВНОГО МИЦЕЛИЯ В РОДЕ AGARICUS L.: FR. EMEND. KARST.

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В работе авторы приводят новые характеристики штаммов 17 видов рода Agaricus, которые имеют, по их мнению, таксономическое значение для определения видов этого рода. В частности, это такие признаки, как: наличие пряжек, бесполое спороношение, относительное содержание ДНК и количество ядер в клетках - последнее свойство возможно имеет экологическое значение. Флюоресценция вегетативного мицелия характерна для всех исследованных видов рода Agaricus. Большинство приведенных в работе характеристик ранее у видов рода Agaricus не исследовались. Авторы делают вывод, что использование микроморфологических критериев вегетативного мицелия в комплексе с культуральными признаками дает возможность установить новые таксономические критерии в роде Agaricus, которые также характерны и для других представителей порядка Agaricales.

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