

ABSTRACT

DEVELOPMENTAL STUDIES OF

CLITOCYBE BENEKEI

BIGELOW & SMITH

By

Francis Eugene Nussbaum, Jr.

A multifaceted study was undertaken to elucidate the biology of Clitocybe benekei, a newly recognized species described in 1970, after its role was recognized in the decline of rose production in Mt. Clemens, Michigan, greenhouses. Several aspects of development were investigated: growth of mycelium, germination of basidiospores, spawn media, production of basidiocarps, nutritional requirements, and nuclear phenomena in basidia and developing basidiospores. Toxicity of crude extracts in relation to antitumor activity was evaluated.

Clitocybe benekei mycelium grows rapidly on agar media. Basidiocarp primordium formation is increased by modification of the nutrients in the medium. Stock cultures are stable on 1.5% malt extract agar media and survive prolonged storage under heavy mineral oil.

Basidiospores germinate on several agar media after two to six weeks incubation at 24 C. Stimulation for

germination is enhanced by the mass effect of dense spore deposits and by the presence of growing C. benekei mycelium.

Grain spawn is an excellent source of inoculum for growing C. benekei basidiocarps. Cacao hulls require enrichment to support growth of C. benekei mycelium.

Typical basidiocarps of C. benekei develop profusely on horse manure compost covered with casing soil at 25 C with 80 to 95% relative humidity, aeration, and continuous illumination. Abnormal epileate basidiocarps were produced at 22 C.

Mannose, dextrin and glucose were excellent carbon sources for mycelial growth in a synthetic medium in shake culture. Urea and asparagine were excellent nitrogen sources. No vitamin requirements were demonstrated. Optimum pH for mycelial growth was 6.5, and optimum temperature was 24 C.

Meiosis in the basidium of C. benekei is typical of eucaryotic organisms and has similarities to other recently investigated Hymenomycetes. The haploid chromosome number is three. Basipetal nuclear migrations occur after meiosis before nuclei migrate into basidiospores. Mature basidiospores are typically uninucleate.

Aqueous crude extracts of C. benekei basidiocarps injected intraperitoneally significantly inhibit growth of Crocker mouse Sarcoma 180. No activity of crude extracts against two forms of leukemia in mice was found by the

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Cancer Chemotherapy National Service Center. Toxic side effects develop in mice when undiluted crude extracts are injected.

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DEDICATION

to my wife, Gloria

and

to my parents,

Mr. and Mrs. Francis E. Nussbaum, Sr.

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INTRODUCTION

The appearance of great numbers of basidiocarps of the previously unknown Clitocybe benekei Bigelow & Smith in a Mt. Clemens, Michigan, greenhouse was a surprise for contemporary mycological taxonomists, and the beginning of the development there of this large fleshy mushroom which is unique among the species of Clitocybe in having dark vinaceous brown-colored spores remains an enigma (Bigelow and Smith, 1970). The large size of the mushrooms and their luxuriant development in the rosebeds of the greenhouse for several years prior to 1970 suggested C. benekei might have commercial uses if it could be cultivated (Figures 1 and 2). Nonetheless, this newly described species would be suitable for a morphological study which would contribute to the knowledge of mushroom morphogenesis thus far not thoroughly investigated.

Clitocybe benekei probably belongs phylogenetically to a group of species centered around C. irina, but is distinguished by the dark-colored spore deposit and relatively coarse spore ornamentation. The habitat and manner of basidiocarps are typical for members of section Verruculosae of Clitocybe. Members of this section are often found fruiting in quantity on mulch or compost heaps out of doors (Bigelow & Smith, 1970).



Figure 1. Immature basidiocarps of *C. benekei* growing in greenhouse rosebeds at Mt. Clemens, Michigan.



Figure 2. Mature basidiocarps of *C. benekei* growing in greenhouse rosebeds at Mt. Clemens, Michigan.

Considering the importance of higher Basidiomycetes in forest ecology and to man as food or in medical research, further study of any species of the group should be a meritorious endeavor. The objectives of this study of C. benekei have been to follow its life history from basidiospore to mature basidiocarp giving special attention to spore germination, nutritional requirements, basidiocarp development, and cytological studies. Preliminary investigations of toxicity and tumor inhibiting properties were also made.

LITERATURE REVIEW

The economic importance of the series Hymenomycetes and Gasteromycetes is diverse, and an even greater potential exists for these organisms. Clitocybe benekei was first brought to the attention of mycologists at Michigan State University because of its deleterious effects on rose production (Beneke et al., 1969). Other higher Basidiomycetes are serious pathogens causing rots and other diseases of forest and shade trees. All wood destroying fungi with an occasional exception belong to a few Homobasidiomycete families (Boyce, 1961). Although these organisms cause significant economic loss by decaying lumber and other wood products (Chahal and Gray, 1968), their mechanisms for decaying organic debris can be beneficial (Alexopoulos, 1962). Consequently, they are important contributors to the natural carbon cycle (Bolin, 1970 and Fergus, 1960). Many Homobasidiomycetes form mycorrhizae with conifers and other forest trees, and undoubtedly they contribute significantly to forest industries through this symbiotic relationship (Singer, 1961).

Several species of mushrooms are cultivated for food in various parts of the world (Singer, 1961). In the United States Agaricus bisporus is the only mushroom

cultivated as food in significant amounts (Lambert, 1963), but many wild American mushrooms have potential for commercial production (Singer, 1961), and are sought out by amateur mushroom hunters each year for their excellent flavors (Smith, 1967). In Japan and other oriental countries Lentinus edodes (Shii-take), Flammulina velutipes, Auricularia polytricha, Volvariella bombycina and Pholiota nameko are produced extensively on a commercial basis (Gray, 1970). Cultivation of Pleurotus ostreatus is becoming a new industry for farmers in Hungary (Vessey, 1969a and 1969b). In some parts of the world, notably in central and eastern European countries, several species of wild mushrooms are gathered in the forests and sold in the local markets. The padi straw mushroom, Volvariella volvacea, is most extensively cultivated by Cantonese farmers in China, but to some extent it is grown in Southeast Asia, the East Indies and Madagascar (Singer, 1961).

Homobasidiomycetes have also attracted some attention in experimental cancer chemotherapy. The appropriate literature is reviewed below in part G.

A. Isolation and Maintenance of Stock Cultures

Axenic stock cultures have been obtained for investigational purposes in a variety of ways. Duggar (1905) using Agaricus campestris was probably the first to describe a method for obtaining mycelial cultures by transplanting a

small piece of the internal tissue from a basiodiocarp onto nutrient media. Duggar's technique lead to the availability of pure spawn and laid the foundation for the rise of the commercial mushroom growing industry in the United States. The tissue culture technique has since become a common practice for mycologists desiring pure cultures of Hymenomycetes and Gasteromycete species.

Polysporous cultures were probably first obtained in the late nineteenth century from the germination of Agaricus campestris spore deposits collected on sterile substrates according to Costantin and Matruchot (1894). They received a patent for pure spawn production by this method.

For those species whose basidiospores germinate readily, monosporous cultures may be obtained for investigational and genetics studies. Single spores are placed in tubes of media, or single spore germlings are removed from an agar plate containing a very scattered spore deposit. Monosporous cultures were used in research prior to 1920 for studies of Schizophyllum commune and Coprinus spp. (Mounce, 1920).

Stock cultures may also be obtained by tissue culture from those organisms which form sclerotia. Galleymore (1949) obtained cultures of Lentinus tuber-regium in this manner, and Mounce (1920) obtained spore bearing basidiocarps from sclerotia of Coprinus stercorearius placed in sterile wet sand.

Stock cultures are routinely maintained by several techniques. The most common procedures involve periodic transfer of a portion of the mycelium to new media in tubes or plates. Refrigeration may be used with some species to increase the time between transfers. Other techniques employed with some success involve deep freezing (Carmichael, 1962), lyophilization (Ellis and Roberson, 1968), covering colonies with mineral oil (Stebbins and Robbins, 1949), using special low nutrient media and storing under liquid nitrogen (Hwang, 1968).

B. Spore Germination of Hymenomycetes and Gasteromycetes

The most comprehensive review of the literature pertaining to basidiospore germination was made by Kneebone (1950). He lists 564 species with the authors and the general methods used. The list includes 113 species of Thelephoraceae, 44 species of Hydnaceae, 133 species of Polyporaceae, eight species of Boletaceae, 254 species of Agaricaceae and 12 species of Gasteromycetes. The general methods for germination are: in water, or on common synthetic media with yeast or malt extract added; in extracts of higher plants or plant parts; in extracts of fungus sporophores; in extracts of substrata such as soil, leaf mold, or rotted wood; in extracts of dung; following passage through animal digestive tracts; in harvested filtrate from a fungus grown on liquid media; in the presence of

living yeast; directly on plant parts; directly on wood; following alternate wetting and drying of spores; on common media after the spores have been subjected to temperature treatments.

Fries (1966) summarized the knowledge of chemical factors in the germination of basidiospores. Some manipulations which have encouraged or affected germination of Agaricus campestris spores are: mycelium near spores; the state of maturity of the sporocarp when spores were collected; presence of other germinating spores; chloroform treatments; dichloroethane vapors; 2:3-dimethyl-1-pentene vapors; and isovaleric acid vapors. With certain exceptions ecological groupings of Hymenomycetes and Gasteromycetes follow general patterns. Coprophilous fungi do not usually require external stimulants for germination, and like lignicolous polypore species, often germinate in pure water. Some lignicolous agarics are more difficult to germinate. Humicolous litter decomposers of forest soils generally germinate easily and rapidly, although some are sensitive to ammonium ion concentration. The praeticolous inhabitants of soil and humus in open fields seem to require special conditions for spore germination. Praeticolous Gasteromycetes have been germinated by co-culture with the yeast Rhodotorula mucilaginosa var. sanguinea. The most difficult group seems to be the mycorrhizae forming species.

Co-culture with a yeast has given limited success, as have growing mycelium, fruit body extracts, and pine roots.

More recent reports of successful basidiospore germination techniques include those of Watling (1963, 1971). Dried spores from herbarium material are placed in a welled slide in a moist chamber with saturated atmosphere overnight at 27 C. The gradual imbibing of water is thought to be less detrimental to the colloidal protoplasm than rapid flooding. Species of Bolbitus, Conocybe, Agrocybe and Psilocybe have given positive results with this method.

When spore germlings are to be studied cytologically, spore deposits are made on media overlaid with permeable cellophane membranes. After some spores have germinated the membranes can be removed and carried with the germlings through fixing and staining procedures. Chang (1969) used this procedure with Volvariella volvacea. Direct studies of germinating basidiospores were made with Schizophyllum commune spores "seeded" onto microscope slides coated with 18% gelatin medium (Volz and Niederpruem, 1968).

More recently the phenomenon of dimorphism has been recorded for Basidiomycetes. Scheld and Perry (1970) chemically induced dimorphism in basidiospore germlings. Hexoses sterilized by autoclaving are better germinants for basidiospores of Lenzites saepiaria than hexoses sterilized by filtration. Basidiospores that germinated in the presence of hydroxymethyl furfural, a degradation

product of heated hexose, yielded large rounded bodies that, in some cases, developed as a chain of yeast-like cells. Similar results were obtained by treating basidiospores with phenethyl alcohol.

C. Spawn

The term "mushroom spawn" denotes grain or other materials in which the mycelium of a mushroom is grown for the commercial mushroom grower in the United States (Snell and Dick, 1957), to inoculate the compost in his mushroom beds. The word "spawn" itself is much older than the practice of making grain spawn, and in its original context it means the cottony vegetative mycelium of mushrooms (Ainsworth and Bisby, 1963), which presumably was observed associated with and giving rise to mushrooms growing in nature. When mushroom growing first became a domesticated agricultural practice in the late 1600's, spawn was collected in nature by taking some of the soil in a meadow around where mushrooms were found growing, and inoculating it into prepared horse manure beds. Transferring some compost from a productive mushroom bed to a newly prepared bed was also a common means of obtaining spawn (Duggar, 1905). These primitive methods often proved unreliable and even the best spawns would "run out" and production of mushrooms cease. Ferry (1894) reports Costantin and Matruchot developed a process for making pure spawn for which they

received a patent (Costantin and Matruchot, 1894). The shroud of secrecy surrounding the methods of Costantin and Matruchot in some ways delayed progress of the mushroom growing industry. Duggar's (1905) methods published by the United States Department of Agriculture, however, provided a great impetus. The development of grain spawn was a logical innovation from Duggar's technique. Yet, manure brick spawn and tobacco stem spawn are still used in the British mushroom industry (Atkins, 1966).

D. Literature on Basidiocarp Production

Numerous publications describe the cultivation of basidiocarps in agricultural practices or their production under laboratory conditions. In the Hymenomycetes and Gasteromycetes, apparently coprophilous species are easiest to cultivate, followed by lignicolous and humicolous species. Fructifications of species considered normally mycorrhizal are achieved only with great difficulty in culture. The morphology of basidiocarps produced in culture is often quite deviant from that typical of the species; however, most experiments are considered successful if structures bearing basidia and basidiospores are produced. The physical environment as well as the nutritive substrate are important to achieving the proper balance of contributing factors which predispose an organism to form its perfect stage. A brief summary of the environment, both physical and chemical, accompanies each species listed chronologically in the following compendium (Table 1.).

Table 1. Compendium of literature on the cultivation of basidiocarps of various species of Hymenomycetes and Gasteromycetes in agricultural practice or their production under laboratory controlled conditions.

Author(s), Date	Species and Conditions
Tournefort, 1707	<u>Agaricus campestris</u> mycelium from old infected horse manure was inoculated into freshly prepared horse manure beds, beginning of modern mushroom science, French mushroom growing.
Miller, 1731	<u>Agaricus campestris</u> French method of mushroom growing was introduced in England.
Bradley, 1731	<u>Agaricus campestris</u> cultivation on old thatch in England.
Lundberg, 1754	<u>Agaricus campestris</u> a primitive Swedish method, outdoors and in greenhouses.
Lundberg, 1762	<u>Agaricus campestris</u> Swedish method introduced in Denmark. Horse manure, sheep bedding, indirect sunlight, cover with 10 inches of soil, one inch of grass seed, four inches of a mixture of soil from horse stable and a rotten tree, water frequently with lukewarm water.
Miller, 1769	<u>Agaricus campestris</u> French method of mushroom growing now practiced in Germany.
Dahuron, 1769	<u>Agaricus campestris</u> two primitive cultivation methods in Germany.

Table 1.--Continued.

Author(s), Date	Species and Conditions
Müller, 1791	<u>Agaricus campestris</u> spread mushroom seeds and trimmings in suitable places.
Steen, 1795	<u>Agaricus campestris</u> horse manure and oak shavings inoculated with stems and trimmings.
Miller, 1797	<u>Agaricus campestris</u> horticultural description for cultivation, improvements in French mushroom growing method.
Rothe, 1828	<u>Agaricus campestris</u> French method using flat beds.
Trog, 1837	<u>Agaricus campestris</u> used fat soil and manure, sprinkled with urine; mushrooms after 10 to 12 months.
Bentzien, 1858	<u>Agaricus campestris</u> cultivation at palace at Rosenberg, Denmark. French method.
Labourdette, 1861	<u>Agaricus campestris</u> potassium nitrate and sulphate in soil gave bigger mushrooms.
Rothe, 1868	<u>Agaricus campestris</u> horticultural description of French mushroom growing.
Reese, 1875	<u>Coprinus</u> spp. two species grown on undefined plant substrate.
Reess, 1875	<u>Coprinus stercorarius</u> grown on horse manure decoctions.
Van Tieghem, 1875	<u>Coprinus ephemeroides</u> grown on horse manure decoction.
	<u>Coprinus radiatus</u> grown on horse manure decoction.
Wright, 1883	<u>Agaricus campestris</u> grown in subterranean caves.

Table 1.--Continued.

Author(s) , Date	Species and Conditions
Rothe, 1885	<u>Agaricus campestris</u> horticultural description of French mushroom growing.
Jensen, 1888	<u>Agaricus campestris</u> horticultural description of French mushroom growing.
Costantin, 1891	<u>Nyctalis lycoperdoides</u> grown on undefined plant substrate.
Costantin, 1893	<u>Agaricus campestris</u> change cultivation rooms to keep down diseases.
Costantin & Matruchot, 1898	<u>Tricholoma nudum</u> mycelium inoculated into various soils.
Duggar, 1905	<u>Agaricus campestris</u> spawn making and horticultural practice in U.S.A.
Buller, 1905	<u>Lentinus lepideus</u> on rotting wooden street paving blocks placed in moist chamber in light.
Spaulding, 1905	<u>Lenzites saepiaria</u> mycelium inoculated into cut timber. <u>Schizophyllum commune</u> tubes of sterilized green wood.
Bayliss, 1908	<u>Polystictus versicolor</u> mycelium inoculated into wood blocks.
Long & Harsh, 1918	species of wood-rotting Basidiomycetes on plant extracts agar media. <u>Coprinus atramentarius</u> malt, parsnip <u>Daedalea juniperina</u> corn meal, malt, prune <u>Exidea recisa</u> carrot, corn meal, prune, malt <u>Fomes applanatus</u> malt, parsnip

Table 1.--Continued.

Author(s), Date	Species and Conditions
	<u>Fomes arctostaphyli</u> malt
	<u>Fomes laricis</u> corn meal
	<u>Fomes meliae</u> corn meal, prune
	<u>Fomes pinicola</u> prune, corn meal
	<u>Fomes rimosus</u> carrot, malt, prune
	<u>Fomes robiniae</u> corn meal, prune
	<u>Fomes roseus</u> carrot, parsnip, malt, corn meal, etc.
	<u>Fomes scutellatus</u> malt, prune, corn meal, etc.
	<u>Fomes texanus</u> malt, carrot
	<u>Ganoderma sessile</u> corn meal
	<u>Irpex lacteus</u> beet
	<u>Lentinus lepideus</u> corn meal
	<u>Lenzites saepiaria</u> malt, corn meal, prune, parsnip
	<u>Merulius ambiguus</u> malt, carrot, celery, bean, etc.
	<u>Panus</u> sp. corn meal, malt
	<u>Pleurotus ostreatus</u> malt, carrot, beet, prune, etc.
	<u>Polyporus</u> sp. malt, beet, prune, parsnip, etc.
	<u>Polyporus albidus</u> celery, carrot, bean, malt, etc.
	<u>Polyporus anceps</u> carrot, celery, malt, bean, etc.
Long & Harsh, 1918	<u>Polyporus cinnabarinus</u> malt, carrot, alfalfa, etc.

Table 1.--Continued.

Author(s), Date	Species and Conditions
	<u>Polyporus dryophilus</u> corn meal, potato, prune, etc.
	<u>Polyporus ellisianus</u> carrot, malt, bean, etc.
	<u>Polyporus farlowii</u> malt, carrot, corn meal, etc.
	<u>Polyporus obtusus</u> prune, carrot, corn meal
	<u>Polyporus sulphureus</u> celery, prune
	<u>Polyporus texanus</u> carrot, parsnip
	<u>Polystictus hirsutus</u> carrot, prune, parsnip, etc.
	<u>Polystictus versicolor</u> parsnip, malt
	<u>Poria</u> sp. carrot, prune, corn meal, malt
	<u>Stereum umbrinum</u> corn meal, parsnip, potato, etc.
	<u>Stereum versiforme</u> corn meal, beet, parsnip
	<u>Trametes peckii</u> carrot, prune, parsnip, bean, etc.
	<u>Trametes serialis</u> prune, carrot, malt, bean, etc.
Brodsgaard, 1918	<u>Agaricus campestris</u> Danish mushroom growing practices.
Bruun & Lange, 1920	<u>Agaricus campestris</u> horticultural prac- tices in Denmark.
Mounce, 1920	several species of <u>Coprinus</u> on sterilized dung in glass tubes: <u>C. ephemerus</u> , <u>C.</u> <u>lagopus</u> , <u>C. nivens</u> , <u>C. stellatus</u> , <u>C.</u> <u>sterquilinus</u> , <u>C. curtus</u> . <u>C. stercorarius</u>

Table 1.--Continued.

Author(s) , Date	Species and Conditions
	from sclerotia on dung placed on sterile wet sand. <u>C. cordisporus</u> produced only rudimentary fruit bodies.
Mounce, 1922	<u>Coprinus comatus</u> after 10 months on horse dung and sawdust cased with six inches of soil.
Rolfe & Rolfe, 1925	<u>Agaricus campestris</u> in large subterranean caves in England.
Gilmore, 1926	<u>Psilocybe coprophila</u> on sterilized rabbit dung, light required.
Etter, 1929	several species on conifer sawdust enriched with malt extract, corn starch, sphagnum, and corn meal: <u>Trametes peckii</u> , <u>Polyporus arcularius</u> , <u>Coprinus micaceus</u> , <u>Xerotus</u> sp., <u>Pholiota</u> sp., <u>Ganoderma curtisii</u> , <u>Lentinus lepideus</u> , <u>Pleurotus ostreatus</u> , <u>Coprinus atramentarius</u> , <u>Polyporus perennis</u> , <u>Polyporus farlowii</u> , <u>Ganoderma polychromum</u> .
Lambert, 1932	<u>Agaricus campestris</u> practical cultivation methods in U.S.A.
Reitsma, 1932	<u>Armillaria mellea</u> 18 to 22 C, pH 5.0, peptone medium.
Findley, 1932	<u>Lenzites saepiararia</u> apple juice agar. <u>Paxillus panuoides</u> Fr. 20 C, light 2% malt extract agar with 1% malic acid, or liquid turnip juice with malic acid.
Lambert, 1933	<u>Agaricus campestris</u> CO ₂ accumulation gives short stems and small pilei.

Table 1.--Continued.

Author(s) , Date	Species and Conditions
Borriss, 1934	<u>Coprinus lagopus</u> 25 C, blue light.
Hawker, 1936	several species on potato and lentil extracts agar in tubes at room temperature in light: <u>Armillaria mellea</u> (rudimentary) <u>Collybia velutipes</u> , <u>Fomes fraxineus</u> , <u>Hydnum coralloides</u> , <u>Schizophyllum commune</u> , <u>Sphaerobolus stellatus</u> .
Biggs, 1938	five species of Thelephoraceae, conditions not given: <u>Corticium coronilla</u> , <u>Odontia setigera</u> , <u>Peniophora candida</u> , <u>P. ludoviciana</u> , <u>Stereum fuscum</u> .
Cayley, 1938	<u>Agaricus campestris</u> a historical review of the cultivated mushroom.
Lambert, 1938	<u>Agaricus campestris</u> practical cultivation procedures.
Ware, 1938	<u>Agaricus campestris</u> practical cultivation procedures.
Akai, 1940	<u>Collybia velutipes</u> light required.
Badcock, 1941	eight species on enriched sawdust: <u>Trametes serialis</u> , <u>Poria xantha</u> , <u>P. vaporaria</u> , <u>P. vaillantii</u> , <u>Lenzites trabea</u> , <u>Lentinus cochleatus</u> , <u>Phellinus cryptarum</u> , <u>Polystictus versicolor</u> .
Johnson & Jones, 1941	<u>Coprinus cubensis</u> beef extract, peptone, or potato dextrose agar media.
Keyworth, 1941	<u>Coprinus ephemerus</u> glucose and salts, thiamine raises optimal concentration of glucose.

Table 1.--Continued.

Author(s), Date	Species and Conditions
Hawker, 1942	<u>Collybia velutipes</u> 20 to 25 C glucose media, thiamine, KNO ₃ , salts, agar. <u>Hydnum coralloides</u> thiamine required. <u>Schizophyllum commune</u> thiamine required.
Mader, 1943	<u>Agaricus campestris</u> accumulated volatile metabolites reduce fruiting.
Nishikado & Miyawaki, 1943	<u>Pleurotus ostreatus</u> on wood.
Badcock, 1943	the following 82 species of wood decay fungi grown on enriched sawdust in closed laboratory glassware apparatus with high relative humidity and light. <u>Armillaria mucida</u> (Schrad.) Fr., <u>Collybia velutipes</u> (Curt.) Fr., <u>Coniophora cerebella</u> Pers., <u>Echinodontium tinctorium</u> E. & H., <u>Fomes annosus</u> Fr., <u>F. clelandii</u> Lloyd, <u>F. conchatus</u> (Pers.) Fr., <u>F. fomentarius</u> (Linn.) Fr., <u>F. fraxineus</u> (Bull.) Fr., <u>F. igniarius</u> (Linn.) Fr., <u>F. lividus</u> Kalchbr., <u>F. melanoporus</u> Mont., <u>F. pinicola</u> (Sw.) Cke., <u>Ganoderma applanatum</u> (Pers.) Pat., <u>G. resinaceum</u> Boud., <u>Hydnum coralloides</u> (Scop.) Fr., <u>H. erinaceus</u> (Bull.) Fr., <u>Hypholoma sublateritium</u> Schaeff., <u>Irpex obliquus</u> (Schrad.) Fr., <u>Lentinus cochleatus</u> (Pers.) Fr., <u>L. lepideus</u> Fr., <u>Lenzites betulina</u> (Linn.) Fr., <u>L. striata</u> Swartz, <u>L. trabea</u> Pers., <u>Merulius lacrymans</u> (Wulf.) Fr., <u>M. serpens</u> (Tode) Fr., <u>M. tremellosus</u> (Schrad.) Fr., <u>Panus</u>

Table 1.--Continued.

Author(s), Date	Species and Conditions
	<u>torulosus</u> (Pers.) Fr., <u>Paxillus panuoides</u> Fr., <u>Phellinus cryptarum</u> Karst., <u>Pholiota</u> <u>adiposa</u> Fr., <u>P. aegerita</u> (Porta) Fr., <u>P.</u> <u>mutabilis</u> (Schaeff.) Quél., <u>P. squarrosa</u> (Mull.) Fr., <u>Pleurotus euosmus</u> (Berk.) Cke., <u>P. lignatilis</u> Fr., <u>P. ostreatus</u> (Jacq.) Fr., <u>P. palmatus</u> (Bull.) Quél., <u>P. ulmarius</u> Bull., <u>Polyporus anceps</u> Peck, <u>P. balsameus</u> Peck, <u>P. benzoinus</u> (Wahl.) Fr., <u>P. borealis</u> (Wahl.) Fr., <u>P. dichrous</u> Fr., <u>P. frondosus</u> Fr., <u>P. fumosus</u> (Pers.) Fr., <u>P. giganteus</u> (Pers.) Fr., <u>P. gilvus</u> Schw., <u>P. obtusus</u> Berk., <u>P. picipes</u> Fr., <u>P. radiatus</u> (Sow.) Fr., <u>P. rugulosus</u> Lév., <u>P. schweinitzii</u> Fr., <u>P. schoreae</u> Wakefield, <u>P. sulphureus</u> (Bull.) Fr., <u>P. tuckahoe</u> (Gussow) Sac. & Trott, <u>P. tulipiferus</u> (Schw.) Overholts, <u>Polystictus abietinus</u> (Dicks.) Fr., <u>P. cinnabarinus</u> (Jacq.) Cke., <u>P. hirsutus</u> (Wulf.) Fr., <u>P. leoninus</u> Klotz., <u>P. pergamenus</u> Fr., <u>P. sanguineus</u> (L.) Mey, <u>P. versicolor</u> (Linn.) Fr., <u>Poria obducens</u> (Pers.) Fr., <u>P. Rickii</u> , <u>P. subacida</u> Peck, <u>P. Yaillantii</u> (DC.) Fr., <u>P. vaporaria</u> (Pers.) Fr., <u>P. xantha</u> Lind., <u>Schizophyllum commune</u> Fr., <u>Stereum</u> <u>hirsutum</u> Fr., <u>S. illudens</u> Berk., <u>S.</u> <u>lobatum</u> Fr., <u>S. purpureum</u> Fr., <u>S. rugosum</u> (Pers.) Fr., <u>S. sanguinolentum</u> (Alb. &

Table 1.--Continued.

Author(s) , Date	Species and Conditions
	Schw.) Fr., <u>S. spadiceum</u> Fr., <u>Trametes rubescens</u> (Alb. & Schw.) Fr., <u>T. serialis</u> Fr., <u>T. violacea</u> Lloyd.
Treschow, 1944	<u>Psalliota bispora</u> on compost, yield increased by biotin and thiamine. REVIEW.
Voderberg, 1948	<u>Coprinus lagopus</u> thiamine, good carbon sources: cellulose, glucose, maltose, fructose, starch; good nitrogen sources: organic, ammonium, and nitrate nitrogen.
Brodie, 1948	<u>Cyathus stercoreus</u> horse dung, or synthetic medium plus yeast extract.
Borzini, 1949	<u>Agaricus campestris</u> synthetic media.
Gallegmore, 1949	<u>Lentinus tuber-regium</u> loam soil, or potato dextrose agar, light required.
Voderberg, 1949	<u>Coprinus lagopus</u> horse manure in small glass containers.
Ehrlich & McDonough, 1949	<u>Schizophyllum commune</u> mineral-dextrose-vitamin agar.
Lambert & Ayers, 1950	<u>Agaricus bisporus</u> vitamins increased yields on compost.
Lange, 1952	13 species of <u>Coprinus</u> on modified Kauffman's medium: agar, maltose, peptone, salts, horse dung decoction. <u>C. angulatus</u> , <u>C. plagioporus</u> , <u>C. hiascens</u> , <u>C. sclerocystidiosus</u> , <u>C. stellatus</u> , <u>C. pellucidus</u> , <u>C. heptemerus</u> , <u>C. bisporus</u> , <u>C. sassii</u> , <u>C. heterosetulosus</u> , <u>C. congregatus</u> , <u>C. ephemerus</u> , <u>C. hexagonosporus</u> ,

Table 1.--Continued.

Author(s) , Date	Species and Conditions
Reeve, 1952	<u>Agaricus campestris</u> on compost, vitamins increased yield.
Plunkett, 1953	<u>Collybia velutipes</u> light, pH 5.2 to 7.2 sucrose and glucose good carbon sources; asparagine and ammonium tartarate good nitrogen sources.
Bille-Hansen, 1953a	<u>Coprinus sassii</u> on media with compost or horse dung extracts; synthetic media with thiamine, maltose, mineral salts (nitrate); asparagine inhibits fructification.
Bille-Hansen, 1953b	<u>Coprinus sassii</u> synthetic medium with glucose or sucrose. <u>C. congregatus</u> synthetic medium with maltose <u>C. heptemerus</u> synthetic medium with maltose
Bukowski, 1954	<u>Volvariella volvacea</u> developed from casing soil in mushroom beds.
Aschan, 1954a	<u>Collybia velutipes</u> 2.5% malt extract agar.
Aschan, 1954b	<u>Collybia velutipes</u> pH 4.6 to 7.0, 25 C in dark then 15 C in light; good nitrogen sources, asparagine and ammonium tartarate.
Schneiderhören, 1954	<u>Coprinus lagopus</u> blue light required.
Madelin, 1956a	<u>Coprinus lagopus</u> thiamine required; good carbon sources: cellulose, glucose, maltose, fructose, and starch; good nitrogen sources: organic, ammonium and nitrate.

Table 1.--Continued.

Author(s), Date	Species and Conditions
Madelin, 1956b	<u>Coprinus lagopus</u> 25 C, light required.
Plunkett, 1956	<u>Collybia velutipes</u> CO ₂ reduces pileus expansion, light required. <u>Polyporus brumalis</u> 4% malt extract, stipe elongation occurs as light is reduced.
Bonner et al., 1956	<u>Agaricus campestris</u> 18 to 20 C, small commercial flats, high relative humidity.
Urayama, 1957	<u>Psilocybe panaeoliiformis</u> stimulation by specific bacteria.
Scheler-Correns, 1957	<u>Coprinus lagopus</u> good nitrogen sources: asparagine, glutamic acid, potassium nitrate, glycine, alanine, aspartic acid, and valine.
Raper & Krongelb, 1958	<u>Schizophyllum commune</u> light, glucose media, thiamine required, stimulated by varied chemicals, genetic basis for abnormal forms.
Koch, 1958	<u>Polystictus versicolor</u> on a synthetic medium with glucose, alanine, salts and thiamine; light required. <u>Pleurotus ostreatus</u> on a synthetic medium with cellulose, peptone, salts and thiamine, light. <u>Psalliota bispora</u> light not required, sterilized horse manure.
Hoffman & Hunt, 1958	<u>Agaricus bisporus</u> on compost, much greater yield when riboflavin, nicotinic acid and pantothenic acid combination is added.

Table 1.--Continued.

Author(s), Date	Species and Conditions
Block, Tsao & Han, 1958	<u>Agaricus campestris</u> composted gumwood sawdust. <u>Pleurotus ostreatus</u> balsa sawdust plus oatmeal or soybean meal.
Aschan-Aberg, 1958	<u>Collybia velutipes</u> 25 C in dark then one week at 15 C in light, pH 5.2 to 7.2.
Plunkett, 1958	<u>Polyporus brumalis</u> 4% malt extract solution, glass apparatus, 20 C, light.
Bevan & Kemp, 1958	<u>Collybia veletipes</u> regenerated from stipes on malt extract agar.
Tamblyn & DaCosta, 1958	several species on enriched sawdust in glass jars with a wood block in light, humid non-stagnant atmosphere. <u>Schizophyllum commune</u> , <u>Fomes gilvus</u> , <u>Polyporus</u> sp(p)., <u>Coriolus</u> sp(p)., <u>Trametes</u> sp(p)., <u>Hexagona</u> sp(p)., <u>Fomes</u> sp(p)., <u>Ganoderma</u> sp(p)., <u>Elfvingia</u> sp(p)., <u>Poria</u> sp(p)., <u>Daedalea</u> sp(p)., <u>Merulius</u> sp(p)., <u>Corticium</u> sp(P)., <u>Peniophora</u> sp(p)., <u>Coniophora</u> sp(p)., <u>Stereum</u> sp(p)., <u>Hydnum</u> sp(p)., <u>Hymenochaete</u> sp(p)., <u>Odontia</u> sp(p)., <u>Lenzites</u> sp(p)., <u>Lentinus</u> sp(p)., <u>Paxillus</u> sp(p)., <u>Hypholoma</u> sp(p).
Block, Tsao & Han, 1959	<u>Pleurotus ostreatus</u> composted sawdust in small wooden trays; 75 to 85% relative humidity gives normal sporophores, abnormal development with 95 to 100%

Table 1.--Continued.

Author(s) , Date	Species and Conditions
	relative humidity; pH 5.0 to 6.0; synthetic medium with thiamine.
Robbins & Hervey, 1959	<u>Poria ambigua</u> blue light, glucose media, thiamine.
Passecker, 1959	<u>Psalliotia augusta</u> Fr. on horse manure with casing soil. <u>P. pseudarvensis</u> Pass. is cultivated on grass plots by Austrian inhabitants. <u>Tricholoma japonicum</u> on bedlogs of European beech.
	<u>Pleurotus ostreatus</u> on beech wood; light required.
Peterson, 1960	<u>Coprinus ephemerus</u> asparagine and thiamine required.
Denyer, 1960	<u>Flammula alnicola</u> on soil, aeration, higher temperature then reduced temperature. <u>F. conissans</u> reduced temperature on wood, high humidity, still atmosphere.
Robbins & Hervey, 1960	<u>Poria ambigua</u> dextrose, casein hydrolysate, salts, B-vitamins, 25 C, blue light.
Aschan-Aberg, 1960a	<u>Collybia velutipes</u> steady 20 C is upper limit for normal basidiocarp formation.
Aschan-Aberg, 1960b	<u>Collybia velutipes</u> blue light.
Madelin, 1960	<u>Coprinus lagopus</u> synthetic medium of glucose, alanine, salts and thiamine, light.

Table 1.--Continued.

Author(s), Date	Species and Conditions
Plunkett, 1961	<u>Polyporus brumalis</u> 4% malt extract agar, light required, initial pH 4.2 to 4.3.
Alicbusan & Ela, 1961	<u>Volvariella volvacea</u> 30 to 45 C, ipil-ipil leaves or wheat bran or dried grain, etc.
Pantidou, 1961	<u>Phlebopus sulphureus</u> conditions not given.
Yurchenco & Warren, 1961	four species of <u>Hericium</u> on enriched sawdust with aeration and high humidity. <u>Hericium caput-ursi</u> , <u>H. erinaceus</u> , <u>H. coralloides</u> , <u>H. septentrionale</u> .
Long, 1962	<u>Collybia velutipes</u> light required, CO ₂ reduces pileus expansion, young sporocarps inhibited by chloramphenicol and 8-azaquanine.
Niederpruem, 1962	<u>Schizophyllum commune</u> accumulated CO ₂ arrests fruiting.
Kuhlman & Hendrix, 1962	<u>Fomes annosus</u> 20 to 24 C, peptone and 2% ethanol in media.
Pantidou, 1962	<u>Phlebopus lignicola</u> room light and temperature.
Niederpruem, 1963	<u>Schizophyllum commune</u> CO ₂ arrests fruiting, light needed.
Hobbs & Niederpruem, 1963	<u>Schizophyllum commune</u> nitrogen activated homokaryotic fruiting, pH 4.0, 23 C, light; CO ₂ arrests fruiting.
Flexer, 1963	<u>Polyporus palustris</u> thiamine required, CO ₂ inhibits, light required, defined medium.

Table 1.--Continued.

Author(s), Date	Species and Conditions
Alasoadura, 1963	<u>Sphaerobolus stellatus</u> defined medium with starch, 20 C, oatmeal and malt extract agar media in light.
Gruen, 1963	<u>Agaricus bisporus</u> commercial compost flats; lamellae are regulatory center of stipe and pileus growth.
Lambert, 1963	<u>Agaricus bisporus</u> commercial production methods in U.S.A.
Lockard, 1963	<u>Psilocybe cubensis</u> on compost; on rye in Ehrlenmeyer flasks. <u>Panaeolus sphinctrinus</u> commercial-type production. <u>Agaricus bisporus</u> casing soil over compost or grain.
Watling, 1963	several members of Bolbitiaceae in bottles with moist aveolated shredded pulp paper and nutrient agar.
Cauchon, 1963	<u>Conocybe percineta</u> , <u>Bolbitius vitellinus</u> , <u>C. subpubescens</u> , <u>Agrocybe semi-orbicularis</u> . fruit bodies more typical and abundant in 3% malt extract agar slant tubes facing down. <u>Schizophyllum commune</u> , <u>Hohenbuehelia reniformis</u> , <u>Pycnoporus cinnabarinus</u> , <u>Phaeophlebia stigoso-zonata</u> , <u>Lenzites saepiaria</u> .
Niederpruem, Hobbs & Henry, 1964	<u>Schizophyllum commune</u> good carbon sources: sucrose, maltose, trehalose, cellobiose, glucose, fructose, mannose,

Table 1.--Continued.

Author(s) , Date	Species and Conditions
	galactose, mannitol, xylose, glycerol, ethanol; good nitrogen sources: peptone, tryptone, casitone, yeast extract, asparagine, glutamine, glutamic acid, serine, alanine, arginine, urea, ammonium salts.
McLaughlin, 1964	<u>Suillus rubinellus</u> Hagem's medium modified by Modess, 20 to 25 C, fluorescent light.
Pantidou, 1964	<u>Xerecomus badius</u> Hagem-Modess medium. <u>X. illudens</u> Hagem-Modess medium.
Maniotis, 1964	<u>Coprinus clastophyllus</u> (<u>Rhacophyllus lilacinus</u>) tree bark in a moist chamber.
Voelz & Niederpruem, 1964	<u>Schizophyllum commune</u> glucose, asparagine, thiamine, salts, agar.
Kinugawa & Furukawa, 1965	<u>Collybia velutipes</u> two days at 5 to 10 C then 15 C; or alternate 5 and 20 C.
Lu, 1965	<u>Cyathus stercoreus</u> 25 C, light required, Brodie agar medium.
Chang, 1965	<u>Volvariella volvacea</u> rice straw beds.
Wells, 1965	<u>Schizophyllum commune</u> minimal media.
Wessels, 1965	<u>Schizophyllum commune</u> 25 C, light, aeration, synthetic medium.
Toole, 1966	<u>Pleurotus ostreatus</u> inoculated into living oak trees.
Schisler & Sinden, 1966	<u>Agaricus bisporus</u> greater yields with seeds added to casing soil.

Table 1.--Continued.

Author(s), Date	Species and Conditions
Long, 1966	<u>Collybia velutipes</u> modified Plunkett's medium, CO ₂ inhibition of pileus expansion is limited to the expansion phase.
Lu, 1967	<u>Coprinus lagopus</u> light, 25 C, Brodies agar medium.
Wilson & Niederpruem, 1967	<u>Schizophyllum commune</u> glucose minimal medium.
Takemaru & Kamada, 1969	REVIEW
Eger, 1968	<u>Pleurotus</u> sp. stimulated by fruit body extracts of <u>Agaricus</u> or <u>Pleurotus</u> .
Flegg, 1968	<u>Agaricus bisporus</u> on compost in controlled environment cabinets.
Fritsche, 1968	<u>Agaricus bisporus</u> a sterile clump "carpophoroid" mutant is reported.
Manachere, 1968	<u>Coprinus congregatus</u> Bull. 24 C, light, convenient substrates.
Randle, 1968	<u>Agaricus bisporus</u> on sawdust or peat or dried compost or chopped straw plus processed sewage sludge or hoof and horn or urea-formaldehyde or urea in 300 ml beakers.
Rosinski & Robinson, 1968	<u>Panus tigrinus</u> 3% malt extract agar 22 to 24 C. <u>Lentodium squamulosum</u> 3% malt extract agar, 22 to 24 C.
Tsusué & Yanganita, 1968	<u>Coprinus macrorhizus</u> f. <u>microsporus</u> light, potato sucrose agar, pH 6.0.

Table 1.--Continued.

Author(s), Date	Species and Conditions
Moore, 1969	<u>Coprinus lagopus</u> synthetic media.
Alasoadura, 1969	<u>Sphaerobolus stellatus</u> blue light required; maturing fruits react to yellow-red light.
Arita <u>et al.</u> , 1969	<u>Pholiota nameko</u> on sawdust media.
Volz & Beneke, 1969	REVIEW
Chang & Chu, 1969	<u>Volvariella volvacea</u> no fruit bodies at temperatures below 20 C.
Kimura, 1969	<u>Coprinus macrorrhizus</u> f. <u>microsporus</u> on complete media in petri dishes.
Komatsu, 1969a	<u>Pholiota nameko</u> sawdust-ricebran media.
Komatsu, 1969b	<u>Lentinus edodes</u> (Berk.) Sing. on bed-logs.
Flexer, 1969	<u>Polyporus palustris</u> complete medium: glucose, salts, peptone, thiamine, and trace elements.
Michalski & Beneke, 1969	<u>Pleurotus ostreatus</u> 2.5% malt extract agar slants in quart jars.
Morrison, 1969	<u>Poria weirii</u> glucose, salts, asparagine, thiamine; with or without <u>Thuja</u> extracts.
Niederpruem & Wessles, 1969	<u>Schizophyllum commune</u> minimal medium of glucose, asparagine and thiamine; other carbon sources: disaccharides, monosaccharides, xylose, sugar alcohols and

Table 1.--Continued.

Author(s), Date	Species and Conditions
	ethanol; good nitrogen sources; peptone, tryptone, certain amino acids, urea, ammonium salts, CO ₂ inhibits, light required, 20 to 25 C.
Rao & Niederpruem, 1969	<u>Coprinus lagopus</u> glucose, malt extract, yeast extract, agar.
Volz & Niederpruem, 1969	<u>Schizophyllum commune</u> glucose, asparagine, thiamine, salts, agar.
Bigelow, 1970	<u>Clitocybe tenuissima</u> malt extract or PDA with light and agar overlay.
Chang & Ling, 1970	<u>Volvariella volvacea</u> small straw beds in laboratory.
Fordyce, 1970	<u>Agaricus bisporus</u> compost, studied other microorganisms present during preparation and cropping.
Heintz & Neiderpruem, 1970	<u>Coprinus lagopus</u> glucose, yeast extract and malt extract agar.
McLaughlin, 1970	<u>Boletus rubinellus</u> synthetic media, initiation 17 to 29 C, maturation 22 to 25 C, light, aerated chambers, CO ₂ inhibits.
Scheld & Perry, 1970	<u>Lenzites saepiaria</u> details not given for lab culture.
Watling, 1971	several species on malt extract agar with peptone.
	<u>Psilocybe merdaria</u> , <u>P. fimetaria</u> , <u>P. coprophila</u> , <u>Pleurotus ostreatus</u> , <u>Crepidotus variabilis</u> , <u>Clitopilus pinsitus</u> .
San Antonio, 1971	<u>Agaricus bisporus</u> soil cased grain spawn in humid aerated incubators.
Miller, 1971	<u>Lentinellus cochleatus</u> produces different forms at different temperatures.

E. Nutritional Requirements for Mycelial Growth

There are numerous investigations reported in the literature on the nutritional requirements of mycelium of Hymenomycetes and Gasteromycetes. These were extensively reviewed in 1969 by Volz and Beneke who concluded: (1) each species apparently has specific nutrient requirements and combinations for mycelial growth; (2) it is essential that a defined medium be used for nutritional studies; (3) thiamine is the most useful vitamin for mycelial formation; (4) various growth promoting substances do not significantly increase vegetative growth; (5) no carbon or nitrogen compound can singly be identified as most useful for mycelial growth; and (6) the pH and temperature may greatly modify the results of the other parameters. Certain corollaries are also suggested by the references in this review: (1) light, gravity and accumulated CO₂ do not usually critically effect mycelial growth; and (2) nutrients supplied as metallic salts may appear ineffective due to toxic concentrations of certain metallic ions.

F. Cytology

While there may be between 5,000 to 10,000 known species of Hymenomycetes and Gasteromycetes (Ainsworth and Bisby, 1963), only between 300 and 400 of these species have had chromosome numbers determined (Table 2). Certain species have been investigated more than once by different investigators, and conflicting chromosome counts have been reported. The following is a listing of some of the more important published information available on chromosome numbers for Hymenomycete and Gasteromycete species (Table 2).

Table 2. Compendium of reports and reviews of chromosome numbers for Hymenomycete and Gasteromycete species.

Author(s), Date	Species	Haploid Number
Wager, 1892,	<u>Agaricus stercorarius</u>	6 - 8
1893	<u>Amanita muscaria</u>	6 - 8
Maire, 1902	<u>Leptotus bryophilus</u>	2
	<u>Cantharellus cinereus</u>	2
	<u>Hygrocybe conica</u>	2
	<u>H. ceracea</u>	2
	<u>H. agathosmos</u>	2
	<u>H. lucorum</u>	2
	<u>Lactarius deliciosus</u>	2
	<u>Coprinus radiatus</u>	2
	<u>Psathyrella disseminata</u>	2
	<u>Hypholoma appendiculatum</u>	2
	<u>Pholiota lucifera</u>	2
	<u>Paxillus involutus</u>	2
	<u>Amanita pantherina</u>	2
	<u>Clitocybe aurantiaca</u>	2
	<u>Mycena galericulata</u>	2
	<u>Lycoperdon piriforme</u>	2
	<u>L. caelatum</u>	2
	<u>Cyphella ampla</u>	2
	<u>Geaster fimbriatus</u>	2
	<u>Boletus</u> spp.	2
	<u>Fistulina hepatica</u>	2
	<u>Clavaria rugosa</u>	2
Nichols, 1905	<u>Hypholoma perplexum</u>	8 or more
Fries, 1911	<u>Hygrocybe conica</u>	2
Kniep, 1911	<u>Armillaria mellea</u>	4
Wager, 1911	<u>Mycena galericulata</u>	4
	<u>Pholiota praecox</u>	6 - 8
Levine, 1913	<u>Boletus granulatus</u>	3 or 4
	<u>B. castaneus</u>	3 or 4
	<u>B. albellus</u>	3 or 4
	<u>B. vermiculosus</u>	3 or 4
	<u>B. versipellis</u>	3 or 4
	<u>B. chrysenteron</u>	3 or 4

Table 2.--Continued.

Author(s), Date	Species	Haploid Number
Kniep, 1916	<u>Armillaria mucida</u>	at least 4
Juel, 1916	<u>Cantharellus cibarius</u>	ca. 2
	<u>Craterellus cornucopioides</u>	2
	<u>C. sp.</u>	2
	<u>C. sp.</u>	2
	<u>Clavaria aurea</u>	3
	<u>C. ligula</u>	3 or 4
	<u>C. pistillaris</u>	4
Hirmer, 1920	<u>Psalliota perrara</u>	2
Bauch, 1926	<u>Camarophyllus virgineus</u>	2
Kuhner, 1926	<u>Hygrocybe constans</u>	probably 2
Tischler, 1927	REVIEW	
Kuhner, 1928	<u>Lentinus variabilis</u>	2 or more
Sass, 1928	<u>Psalliota campestris</u>	4
Sass, 1929	<u>Coprinus ephemerus</u> f. <u>bispora</u>	8-10
Wakayama, 1930	REVIEW	
Wakayama, 1930	<u>Cortinarius cinamomeus</u> L.	4
	<u>Pholiota sp.</u>	2
	<u>Russula subfoetens</u> W. G. Sm.	4
	<u>Hypholoma fasciculare</u> (Huds.) Fr.	2
	<u>Pleurotus ostreatus</u> Jacq.	4
	<u>Coprinus micaceus</u> B.	4
	<u>Mycena haematopoda</u> Pers.	6
	<u>Tricholoma rutilans</u> Schaeff.	2
Wakayama, 1932	<u>Cantharellus minor</u> Peck	6
	<u>Clitocybe laccata</u> (Scop.) Fr.	6
	<u>Collybia radicata</u> Relh.	4
	<u>C. velutipes</u> R. Karst.	4
	<u>Coprinus atramentarius</u> (Bull.) Fr.	4
	<u>Cortinellus shiitake</u> P. Henn.	6
	<u>Hypholoma appendiculatum</u> Bull.	6

Table 2.--Continued.

Author(s), Date	Species	Haploid Number
	<u>Inocybe rimosa</u> (Bull.) Fr.	4
	<u>Lactarius akahatsu</u> Tanaka	6
	<u>L. vellereus</u> Fr.	6
	<u>Mycena galericulata</u> Scop.	4
	<u>Psathyrella disseminata</u> (Pers.) Fr.	4
	<u>Russula emetica</u> (Schaeff.) Fr.	4
	<u>R. fragilis</u> (Pers.) Fr.	4
	<u>R. fragilis</u> var. <u>nivea</u> (Pers.) Cooke	6
	<u>R. virescens</u> (Schaeff.) Fr.	4
	<u>Tricholoma melaleucum</u> Pers.	4
	<u>Boletus edulis</u> Bull.	6
	<u>B. luridus</u> (Schaeff.) Fr.	4
	<u>B. luteus</u> L.	4
	<u>Fistulina hepatica</u> (Huds.) Fr.	4
	<u>Polyporus squamosus</u> (Huds.) Fr.	4
	<u>Polystictus cinabarius</u> (Jacq.) Fr.	4
	<u>Strobilomyces strobilaceus</u> (Scop.) Berk.	4
	<u>Clavaria botrytis</u> Pers.	4
	<u>C. cristata</u> (Holmsk.) Pers.	4
	<u>C. inaequalis</u> (Mull.) Quel.	4
	<u>C. mucida</u> Pers.	4
	<u>C. purpurea</u> Fr.	4
	<u>Geaster hygrometricus</u> Pers.	2
	<u>Lasiosphaera fenlzii</u> Reichardt	4
	<u>Lycoperdon gemmatum</u> Batch	2
	<u>Craterellus cornucopioides</u> Pers.	4

Table 2.--Continued.

Author(s), Date	Species	Haploid Number
Vokes, 1931	<u>Coprinus atramentarius</u>	4
Lander, 1933	<u>Schleroderma lycoperdoides</u>	2
Lorenz, 1933	<u>Sphaerobolus tubulosus</u>	3 or 4
Chow, 1934	<u>Coprinus hendersonii</u>	2
	<u>C. lagopus</u>	2
	<u>C. tomentosus</u>	2
Colson, 1935	<u>Psalliota campestris</u>	9
Lander, 1935	<u>Pisolithus tinctorius</u>	4
Goto, 1936	<u>Corticium rolfsii</u>	4
Whelden, 1936	<u>Peniophora</u> sp.	4 or 8
Brandza & Solacolu, 1937	<u>Geastrum fimbriatum</u>	2
	<u>Calvatia candida</u>	2
Kemper, 1937	<u>Coniophora puteana</u>	4
	<u>C. arida</u>	4
Gries, 1937a	<u>Lepiota acutesquamosa</u>	6
Gries, 1937b	<u>Tylostoma mammosum</u>	4
Salmon, 1937	<u>Lactarius vellereus</u>	4
Sarazin, 1938	<u>Psalliota campestris</u>	4
Ritchie, 1941	<u>Russula emetica</u>	4
Hagerup, 1945	<u>Lepiota lenticularis</u>	4
Ritchie, 1948	<u>Amanita caesarea</u>	4
Ehrlich & McDonough, 1949	<u>Schizophyllum commune</u>	3
Olive, 1953	REVIEW	
Heim, 1954	<u>Lycoperdon piriforme</u>	6
	<u>Panus torulosus</u>	6
	<u>Clitocybe brumalis</u>	8
	<u>C. cerrussata</u>	8
	<u>C. nebularis</u>	8

Table 2.--Continued.

Author(s), Date	Species	Haploid Number
Kimura & Takemaru, 1955	<u>Coprinus machrorhisus</u> Rea var. <u>microsporus</u> Hongo	4
Evans, 1956	<u>Agaricus campestris</u> var. <u>bisporus</u>	12
Evans, 1959	<u>Agaricus campestris</u> var. <u>bisporus</u>	12
Sarkar, 1959	<u>Ganoderma lucidum</u> (Leyes ex Fr.) Karst.	5
Banerjee & Samadder, 1960	<u>Daedalea flavida</u> Lev.	6
Naha, 1960	<u>Trametes cingulata</u> Berk.	6
Ward & Ciurysek, 1961	Unidentified Basidiomycete	4
Banerjee & Banerjee, 1961	<u>Trametes lactinea</u> Berk.	3
Hughes, 1961	<u>Agaricus campestris</u>	12
Banerjee & Nandi 1962	<u>Flammulina dilepis</u> B. & Br.	6
Lu & Brodie, 1962	<u>Cyathus stercoratus</u>	12
Olive, 1962	REVIEW--373 species of Hymenomycetes and Gasteromycetes	
Lu, 1964	<u>Cyathus olla</u> Pers.	12
Dowding & Bulmer, 1964	<u>Calvatia gigantea</u>	3
	<u>Lycoperdon curtisii</u>	4
	<u>L. pusillum</u>	3
	<u>L. pyriforme</u>	4
Keeping & Bulmer, 1964	<u>Calvatia gigantea</u>	3

Table 2.--Continued.

Author(s) , Date	Species	Haploid Number
	<u>Lycoperdon curtissii</u>	4
	<u>L. pusillum</u>	3
	<u>L. pyriforme</u>	4
Duncan & MacDonald, 1965	<u>Marasmius androsaceus</u> (L. ex Fr.) Fr.	4
	<u>M. rotula</u> (Scop. ex Fr.) Fr.	4
Olive, 1965	REVIEW	
Lowry, unpublished (cit. Olive, 1965)		
	<u>Amanita fulva</u>	8
Mc Claren, 1967	<u>Coprinus atramentarius</u> Fries	3
Wilson <u>et al.</u> , 1967	<u>Fomes annosus</u>	7
Huffman, 1968	<u>Collybia maculata</u> var. <u>scorzonia</u>	8
Huffman, 1969	<u>Collybia maculata</u> var. <u>scorzonia</u>	8
Harmaja, 1969	<u>Clitocybe phyllophila</u> var. <u>phyllophila</u>	3

G. Tumor-inhibition, Toxicity, and other Pharmacological
Properties of Hymenomycetes
and Gasteromycetes

During the 1940's and 1950's Basidiomycetes like other fungi and microorganisms were intensively investigated in a search for new antibiotics. Several antibiotic substances were found from a variety of species including Clitocybe illudens (Anchel et al., 1950 and 1952). Wilkins (1945, 1946a, 1946b, 1947a, 1947b, 1947c, 1948) screened over 500 species of higher Basidiomycetes. A more complete review of the literature pertaining to antibiotics produced by Basidiomycetes was made by Stevens (1957).

The earliest reported species in which antitumor activity was shown were Boletus edulis, Calvatia bovista, Calvatia craniformis, Calvatia gigantea, Calvatia cyathiformis and Collybia radicata (Lucas et al., 1957; Lucas et al., 1959; and Stevens 1957). Later Yurchenco and Warren (1961) reported Hericium erinaceus showed activity against transplantable tumor in mice. Beneke (unpublished data) has repeatedly demonstrated activity against Sarcoma 180 in mice by extracts of Suillus luteus, while Burton and Cain (1959) reported antileukaemic activity by polyporic acid which is found in a variety of fungi. Clitocybe illudens produces lunamycin an antitumor substance which has been characterized and investigated in Japan (Shirahama et al., 1962a and 1962b) where this species is known as Lampteromyces japonicus.

Tumor inhibiting principles are found in the sporocarps of several Hymenomycetes and Gasteromycetes. Espen-shade and Griffith (1966) tested about 50 different liquid media and screened over 7,000 samples of culture filtrates for activity against transplantable mouse tumors. About 1% of the cultures showed positive activity against one or more tumor types. Three screening systems were used: Sarcoma 180, Carcinoma 755, and Leukemia 1210. Positive activity was associated with certain samples of the following species: Collybia radicata, Coprinus nycthemerus, Corticium rolfsii, Irpex consors, I. flavus, Lentinus lepideus, Lenzites saepiaria, L. trabea, Merulius niveus, Pholiota formosa, Pleurotus passeckerianus, Polyporus distortus, P. obtusus, Poria corticola, P. subacida, P. xantha, Stereum subpileatum, Tricholma panaeolum. None of these were active against Leukemia 1210, but Tricholma panaeolum, Lentinus lepideus and Merulinus niveus were active against Carcinoma 755.

In a similar report (Gregory et al., 1966) anti-tumor activity was shown by: Agaricus sp., Boletus sp., Collybia radicata, Coprinus nycthemerus, Corticium rolfsii, Hydnum erinaceum, Irpex consorus, I. flavus, Lenzites trabea, L. saepiaria, Lentinus lepideus, Merulius niveus, Nidularia sp., Pholiota formosa, Polyporus distortus, P. obtusus, Polyporus sp., Poria corticola, P. subacida, P. xantha, Poria sp., Schizophyllum sp., Stereum subpileatum, Thelephora

sp. and Tricholoma panaeolum. Of these, Merulius niveus, Poria corticola and Tricholoma panaeolum showed activity against Carcinoma 755, and Poria subacida showed activity against Leukemia 1210.

One of the most extensively investigated antitumor products produced by a Basidiomycete was calvacin from Calvatia gigantea (Lucas et al., 1959; Roland et al., 1960; and Beneke, 1963). In reporting results of toxicological studies made prior to clinical trials (Sternberg et al., 1963) stated that calvacin, a potent antitumor mucoprotein had a cumulative effect and caused prolonged intoxication characterized by anorexia and extreme weight loss. Calvacin induced a wide spectrum of lesions in experimental animals including: hepatic necrosis, biliary obstruction, renal tubular necrosis, fibrinoid degeneration, myofibrillar necrosis in cardiac and skeletal muscle, and pulmonary hemorrhages, and the compound was shown to be a complex mixture of antigenic substances with sensitization demonstrated in rabbits, guinea pigs, and dogs. As anticipated, clinical trials were complicated by the antigenic properties of calvacin.

Oxytocins have been reported from Clitocybe subilludens (Murrill, 1953) and ergot-like alkaloids (Foote et al., 1953). Genest and Hughes (1968) recently isolated the hallucinogen muscarine from Clitocybe spp. while atramentin, a smooth muscle stimulant, has been found

in Clitocybe subilludens and in Hydnum diabolus (Sullivan and Guess, 1969; Euler et al., 1965). One species, Clitocybe oleiria, has been shown to be active against leukemia 1210 in mice (unpublished data, Cancer Chemotherapy National Service Center).

In investigations to find botanical sources of influenza inhibitors Cochran, Nishikawa, and Beneke (1966) found activity from extracts or culture filtrates of Calvatia gigantea, Boletus edulis, Amanita muscaria, Lepiota sp., Phallus revenelli, Suillus luteus, Lentinus edulis and Lepiota morgani.

MATERIALS AND METHODS

A. Isolation and Maintenance of Stock Cultures

The stock cultures used in these studies were obtained from a collection of basidiocarps of Clitocybe benekei collected from rose beds mulched heavily with cacao bean hulls in the Floral Avenue Greenhouse in Mt. Clemens, Macomb County, Michigan, on April 3, 1969. Part of this collection was used by Bigelow and Smith (1970) to write the original species description and was designated the holotype for the species.

The large pileus of C. benekei is broadly convex with an incurved margin which becomes wavy to crenate with age. The smooth moist surface is at first buff-colored, but fades to pallid upon drying. The white context does not change overnight when broken or bruised. The lamellae are wood brown at maturity, adnate to shortly decurrent, close, broad, and with thin even edges (Figures 3 and 4). The stipe has a bulbous or equal base, and is solid, fibrous, and white within when young (Bigelow and Smith, 1970). In the cacao bean hull mulch at Mt. Clemens, thick, well developed rhizomorphs were present (Figure 5).



Figure 3. Large, young basidiocarp of C. benekei with light-colored lamellae collected April 3, 1969, at Mt. Clemens, Michigan.



Figure 4. Older basidiocarps of C. benekei with wood-brown lamellae collected April 3, 1969, at Mt. Clemens, Michigan.



Figure 5. Rhizomorphs of C. benekei in cacao bean hulls mulch at Mt. Clemens.

Stock cultures of C. benekei were obtained by removing small pieces of the pileus context tissue under sterile conditions, and placing them into 1.5% malt extract agar medium in plastic petri plates. Internal stipe tissue from mature specimens was not used as this tissue was often shredded into a fibrous, pithy, porous condition, or the stipe had actually become hollow. The stipe and lamellar tissues were also often damaged and contaminated by the feeding activities of slugs, insects, and insect larvae. On 1.5% malt extract agar a good outgrowth of hyphae developed from the tissue transplants within one week. Microscopic examination of this hyphae revealed the presence of clamp connections, and the new colonies were presumed to be C. benekei. Different strain numbers were designated for the colonies which developed from tissue transplants from different basidiocarps. The new strains for cultural studies were maintained on 1.5% malt extract agar medium in plastic petri plates at a constant temperature of 24 C. At monthly intervals a 0.5 cm² block of agar was cut from the edge of an established colony and transferred to a new agar medium plate.

A set of stock cultures was inoculated into test tubes containing 1.5% malt extract agar and another set of cultures was inoculated into test tubes containing medium A (Stevens, 1957). Three weeks after the colonies developed in the tubes, one inch of autoclaved heavy mineral

oil was added for prolonged storage (Stebbins and Robbins, 1949).

B. Spore Germination

Experiments were performed to determine if the basidiospores of Clitocybe benekei could be germinated, and if they could, to attempt to isolate single spore germlings which would develop into monosporous stock cultures for cytological and genetics studies.

In the first experiments spore deposits were collected in petri plates on a variety of agar media, by suspending a section of lamella over the petri plate. The lamellar sections were attached to the inside of the petri plate lids by a petroleum jelly. This gave a compact spore deposit directly under the lamellar section on the surface of the medium. After the deposits were made, the petri dish lids were replaced with new sterile ones, and the plates were incubated at 24 C. The following agar media were used: 2.5% malt extract, 4% corn meal, chlamydospore (Nickerson and Mankowski, 1953), Emmon's (Difco, 1968), Sabouraud's (Beneke and Rogers, 1971), MP5 (Alexopoulos and Beneke, 1962), nutrient (Difco, 1953), BHI (Beneke and Rogers, 1971), and Hagem-Modess (Modess, 1941).

In later experiments spore germinations of C. benekei basidiospores were performed using disposable plastic petri plates containing 1.5% malt extract agar.

Sections of lamellae from a freshly harvested mature mushroom were attached to the bottom inside of a sterile 800 ml or 1000 ml beaker with petroleum jelly, and the beaker was inverted over an opened petri plate so that the spores were directly discharged onto the agar surface. An evenly scattered spore deposit was obtained. The entire apparatus was placed on paper toweling soaked with a 0.5% amaryl solution which minimized contamination and increased the humidity inside the discharge chamber to a level necessary for spore discharge. A thin covering of spores was desirable with a distance of several spore diameters between proximal spores. This distance was necessary to recognize individual germinating spores. Each plate was estimated to have received from one to six million spores. In some experiments on the day following the deposit of spores on the medium, a thin layer of 1.5% liquid malt extract agar at 45 C was poured over them. The plates were incubated at 24 C in an inverted position, and observed periodically under low power of a compound microscope.

C. Spawn Production

Grain spawn was prepared by methods similar to the contemporary method for preparing commercial grain spawn of Agaricus bisporus (Rettew, 1948, Lockard, 1963, Volz, 1966). Rye grain and oats grain were used to make spawn for mushroom production of Clitocybe benekei. After

removing pieces of straw, ergots, foreign grains and seeds, and other debris, the grain was soaked for one or two days in water until fully imbibed. Two hundred fifty ml Ehrlenmeyer flasks were filled half full with imbibed grain (Figure 6), and water was added to half the depth of the grain. In some cases 0.5 g CaCO_3 was added per flask. The flasks were covered with four inch aluminum foil squares and autoclaved 30 min. On the following day a 1.0 cm^2 agar block was cut aseptically from a petri plate culture, and transferred to the surface of the autoclaved grain in the flask. Hyphae grew out from the inoculation block and colonized the moist grain within two to three days.

The possibility of using cacao bean hulls as a spawn substrate was also investigated. Cacao bean hulls are marketed as a by-product of chocolate production by the Hershey Company, Hershey, Pennsylvania, for garden mulch under the trade name of Ko-K-O, and were obtained from a local nursery and garden supply dealer. Ko-K-O hulls were soaked two days in water that became dark chocolate brown colored. Some of this water extract was combined with 1.5% agar, sterilized, and poured into petri plates. Clitocybe benekei was inoculated onto the medium from stock cultures and failed to develop indicating incomplete nutrition in the medium. Three experimental groups containing soaked Ko-K-O hulls were tested for use

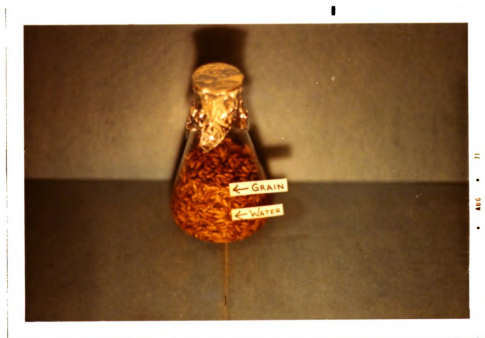


Figure 6. Grain ready to be autoclaved and then inoculated for use as spawn for C. benekei basidiocarp production.

as spawn. Group A consisted of 10 half filled 250 ml Ehrlenmeyer flasks containing only soaked Ko-K-O. Group B consisted of 10 pint size mason jars filled with soaked Ko-K-O to which 100 ml of the following nutrient solution was added: 1.0 g NH_4Cl , 1.0 g KH_2PO_4 , 1.0 g maltose, and 1.0 liter distilled water. Group C consisted of 10 pint size mason jars filled with soaked Ko-K-O to which 100 ml of the following nutrient solution was added: 1.0 g urea, 1.0 g CaCO_3 , 5.0 g glucose, and 1.0 liter distilled water. All jars and flasks were autoclaved 30 min, cooled, and inoculated with C. benekei strain CB-69-01 from stock cultures. After one week the inoculation blocks of groups B and C were "fuzzy" white with emergent hyphae slowly invading the substrate. Group A, the unenriched Ko-K-O, had no emergent hyphae. The flasks in group A were re-inoculated, and one week later there was still no outgrowth of hyphae. At this time each flask in group A was given 0.5 g glucose and 0.5 g CaCO_3 , was shaken to dissolve these additives, was re-autoclaved, cooled, and again inoculated. After one week hyphae were growing from the inoculation blocks into the substrate.

D. Basidiocarp Production of Clitocybe benekei

Basidiocarp production on compost

Basidiocarps of Clitocybe benekei were most successfully produced by a method similar to current practices

used for commercial production of Agaricus bisporus (Lambert, 1963, Atkins, 1966, Richards, 1960, Rettew, 1948, and Gray, 1970). The major differences were the use of significantly higher temperatures, and the use of continuous light. The commercially prepared compost for mushroom production was supplied by a commercial grower. The mushrooms were grown in wooden boxes one ft by one ft by eight inches. These boxes were autoclaved for one hour, removed from the autoclave, and covered with plastic covers in order to reduce air borne contamination while being transported to the commercial mushroom house to be filled with compost. Likewise, filled trays were covered again with plastic covers until returned to the laboratory for the addition of spawn. Each box was filled six inches deep with pasteurized compost. One 250 ml Ehrlenmeyer flask of spawn was thoroughly mixed into the compost of each box. The spawned compost was packed lightly to prevent dessication and to promote contact of the compost with the spawn. Two facilities were available to provide the temperature control, high relative humidity, and aeration necessary for mushroom production. One was an experimental mushroom house in which the relative humidity was maintained between 80% and 90% and the temperature was 22 C. The other was a Sherer controlled environment chamber, model CEL 25 - 7HL where relative humidity exceeded 95% and the temperature was 25 C. Occasional variations and fluctuations in

temperature and humidity occurred in both facilities. Both facilities provided continuous fluorescent lighting and circulating air. The compost was watered as necessary and the surfaces were not permitted to dry out. Six 0.5 inch diameter holes in the bottoms of the boxes prevented the accumulation of excess water. Growth was evident two days after the spawn was placed in the compost, and the mycelium developed extensively throughout the compost by 18 days. On day 18 (day 21 in the second group) an inch of autoclaved casing soil was placed over the surface of the compost. The casing soil had been collected from the forest floor of a campus woodlot, spread 0.5 inch thick on aluminum foil pans, autoclaved one hour, and cooled before use. Mature mushrooms were harvested by pulling up the complete basidiocarp. Fresh weight of the harvested mushrooms was recorded. Mushrooms grown on compost with casing soil were used in various other experiments.

In the first two experimental groups, certain boxes in the experimental mushroom house where the temperature was maintained at 22 C produced abnormal basidiocarps. For the third compost experiment, the temperature of the controlled environment chamber was also reduced to 22 C at spawning and maintained there until the end of cropping.

Basidiocarp production without
compost

Several methods other than using compost and casing soil in one ft by one ft by eight inches wooden boxes were used in attempts to obtain basidiocarps of C. benekei.

1. Deep culture dishes containing an inch of partially composted sawdust supplemented with 100 ml of the following nutrient solution were used: 20 g glucose, 40 g potato flakes, one ml stock thiamine solution and 1000 ml distilled water. These culture dishes remained closed after inoculation with 0.5 cm² agar blocks cut from stock culture colonies of strain CB-69-01, and they were incubated in a 24 C constant temperature room under intermittent fluorescent and day light.

2. Several attempts were made to obtain fructifications in disposable petri plates containing these agar media: 1.5% malt extract and 2.5% malt extract which support basidiocarp development by Pleurotus ostreatus, Hagem-Modess (Modess, 1941) medium used by McLaughlin (1964, 1970) and Pantidou (1961, 1962, 1964) to obtain fructifications of Boletus species, corn meal agar, synthetic agar medium and medium A (Stevens, 1957). The plates remained closed and were incubated in a 24 C constant temperature room inside clear plastic bags which prevented their rapid drying out.

3. Battery jars were placed over some petri dishes which were opened when primordia began to develop in them. Cultures on medium A, malt extract and synthetic agar media were used at various times in attempts to achieve basidiocarp formation. Various schemes were devised to increase humidity inside the battery jars, reduce contamination, and lower the concentration of metabolic carbon dioxide, such as setting the cultures over pans of water or moist toweling, using moist blotter paper liners for the jars, including wells of barium chloride in the jars and adding small amounts of amphyl to the humidifying solutions. Several stock culture strains were used. Cutting sections of colonies growing on malt extract agar and placing them on top of uncut sections as described by Bigelow (1970) for obtaining fructifications of Clitocybe tenuissima was also attempted in the battery jar apparatus.

4. To investigate the hypothesis that conditions which support luxuriant vegetative growth are not the same conditions which bring about good reproductive growth, corn meal agar, was inoculated with several strains of C. benekei in 12 deep culture dishes. The dishes were incubated in a 24 C constant temperature room and received intermittent fluorescent lighting.

5. Six pint mason jars were prepared containing slants of 1.0% malt extract agar. These were covered with

petri dish lids held by wire clamps which allowed raising the lids for atmospheric exchange after colonies became established. After inoculation with strain CB-69-14, the jars were incubated in the 24 C constant temperature room. After two months one jar was placed in the Sherer controlled environment chamber and one jar was placed on the 16 to 18 C cold air vent while another jar was placed at 24 C under continuous fluorescent light.

6. Pint mason jars with slants of 1.5% malt extract agar were inoculated with several strains of C. benekei and incubated in the 24 C constant temperature room for six weeks until primordia began forming. The jars were then filled with autoclaved woodland soil to cover the cultures and returned to the constant temperature room.

7. Six aluminum foil trays nine inches by nine inches by one and a half inches were filled with an inch of woodland soil and autoclaved 30 min. After cooling they were inoculated with several strains of grain spawn and covered with a 10 inch plate glass square before being placed in the Sherer controlled environment chamber at 22 C, with 95% relative humidity and continuous fluorescent lighting. After one week mycelium covered the surface of the soil in the trays and the glass covers were removed. At two weeks four trays were covered with a half inch of autoclaved moist cacao hulls and two trays were covered

with a half inch of sterile woodland soil. After this the temperature was lowered one degree C per day in the growth chamber from 22 C until it was 16 C.

8. In a further attempt to produce basidiocarps several natural substrates were used in deep culture dishes. These natural substrates were enriched with a nutrient solution containing 10 g dextrose, 40 g potato flakes, one ml stock thiamine solution and 1000 ml distilled water. The natural substrates were soaked overnight in the culture dishes in water and then drained the following day. The nutrient solution was added and the culture dishes with enriched media were autoclaved 30 min. Combinations of sawdust, oats and cacao hulls were used (Table 3). Strains CB-69-01, CB-69-02 and CB-69-03 were used to inoculate the cultures (Table 3). After inoculation the cultures were incubated at 24 C under continuous fluorescent illumination. After five weeks they were taken to the experimental mushroom house and the lids opened. After 10 days an inch of sterile casing soil was placed over each culture.

9. Ten pint mason jars of each of the following two substrate combinations were prepared: sawdust and oats (3:1); and sawdust and potato flakes (15 g potato flakes per jar of sawdust). The jars containing (3:1) sawdust and oats were inoculated with strain CB-69-02 whereas the jars containing sawdust and potato flakes

Table 3. Media and stock strains used in deep culture dish experiments for basidiocarp production.

No.	Substrate	Nutrient mls	Strain No.	Spawn Type and Age
187	cacao mulch	150	CB-69-01	oats 7 wk
188	cacao mulch	100	CB-69-01	oats 7 wk
189	cacao mulch	100	CB-69-01	oats 7 wk
190	sawdust:oats (3:1)	100	CB-69-01	oats 7 wk
191	sawdust:oats (3:1)	75	CB-69-02	oats 7 wk
192	sawdust:oats (3:1)	75	CB-69-02	oats 7 wk
193	cacao mulch	100	CB-69-02	oats 7 wk
194	cacao mulch	100	CB-69-02	oats 7 wk
195	cacao mulch	100	CB-69-02	oats 7 wk
196	sawdust	50	CB-69-03	rye 8 wk
197	sawdust	50	CB-69-03	rye 8 wk
198	sawdust	50	CB-69-03	rye 8 wk
199	oats	75	CB-69-03	oats 7 wk
200	oats	75	CB-69-03	oats 7 wk
201	oats	75	CB-69-03	oats 7 wk

were inoculated with strain CB-69-07. These were placed in the 24 C constant temperature room until the mycelium had grown to the bottom of the substrate in the jars. This was five months for the sawdust and oats jars, and four months for the sawdust and potato flakes jars. After the mycelium had developed throughout the jars, they were placed in the Sherer controlled environment chamber with continuous fluorescent lighting, 95% relative humidity and 22 C temperature. To prevent excess water from accumulating in them the jars were placed on their sides.

10. Aluminum foil pans four and a half inches by eight inches by two and a half inches were filled with grain or cacao which had been soaked over night. The pans were covered with aluminum foil and autoclaved 30 min. Several strains were used to inoculate these pans. After inoculation the pans covered with aluminum foil were placed in the Sherer controlled environment chamber at 22 C and 95% relative humidity. After three weeks good mycelial growth had occurred so the foil covers were removed and one half inch of casing soil was placed over some of the cultures in the pans.

E. Nutritional Requirements

The nutritional requirements for submerged shake culture growth of Clitocybe benekei were investigated. A rotary shaking apparatus (Eberbach Corporation #6151) at

150 rpm was used in a constant temperature room at 24 C. The incubation time for experiments was 20 days. Two hundred fifty ml Ehrlenmeyer flasks and all glassware required for the preparation of media were acid washed in aqua regia (18 ml concentrated HNO_3 and 82 ml concentrated HCL acid). A four inch square of aluminum foil was used for a flask cover. Strain CB-69-01 was used for the investigations. The inoculum was grown by the same method as the stock cultures on 1.5% malt extract agar in plastic petri plates for four weeks. Using aseptic technique a 0.25 cm^2 agar block cut from a colony in a petri dish was used to inoculate each flask. The medium in the flasks was cold filter sterilized with 0.1μ filters prior to inoculating. All groups had five replicates. The pH and temperature optimum for mycelial growth, the growth curve, carbon utilization, nitrogen utilization, and vitamin requirements were determined for the organism. A synthetic medium modified from media used for other Basidiomycetes (Lindeberg, 1941, Humfeld and Sugihara, 1952, Fraser and Fujikawa, 1958) was as follows:

The complete synthetic medium contains:

Dextrose	20.0 g
Urea	1.5 g
KH_2PO_4	1.0 g
$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$	0.5 g

MgSO ₄	0.5 g
Fe ^{III} Cl ₃	1.0 ml stock soln.
CaCl ₂	5.0 ml stock soln.
MnCl ₂	1.0 ml stock soln.
Trace elements	10.0 ml stock soln.
Thiamine	1.0 ml stock soln.
Biotin	1.0 ml stock soln.
Pyridoxine	1.0 ml stock soln.
i-Inositol	1.0 ml stock soln.
Glass distilled water	980.0 ml

The stock solutions contain:

Fe ^{III} Cl ₃ ·6H ₂ O	0.1 g / 100 ml H ₂ O
CaCl ₂ ·2H ₂ O	1.5 g / 100 ml H ₂ O
MnCl ₂ ·H ₂ O	1.0 g / 100 ml H ₂ O
Thiamine HCl	10.0 mg/ 100 ml H ₂ O
Crystalline Biotin	0.5 mg/ 100 ml H ₂ O
Pyridoxine HCl	10.0 mg/ 100 ml H ₂ O
i-Inositol	500.0 mg/ 100 ml H ₂ O

The trace element solution contains:

H ₃ BO ₃	0.0001 g
CuSO ₄ ·5H ₂ O	0.001 g
ZnSO ₄ ·7H ₂ O	0.02 g
Na ₂ MoO ₄ ·2H ₂ O	0.0002 g
CoSO ₄ ·7H ₂ O	0.0001 g

Glass distilled water was used to prepare all stock solutions. The stock solutions were stored in a refrigerator at four C in acid washed prescription bottles. Fresh vitamin solutions were prepared every six months. The pH was adjusted to 6.0 before filter sterilizing with six N HCl or six N NaOH.

After the growth period the mycelium was vacuum filtered with a Buchner porcelain funnel onto a piece of Sargent 7.5 cm diam no. 501 filter paper which had previously been dried and weighed. The mycelium retained on the filter paper was placed on a six inch diam watch glass and oven dried at 85 C for about 18 hours. Dry weight of mycelium was determined and the pH of the culture filtrates was determined at the end of the growth periods.

Optimum pH for mycelial growth

Several experiments were performed to determine the optimum pH for shake culture growth of C. benekei CB-69-01 mycelium.

In a preliminary experiment eight groups of five flasks each were prepared with pH values 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0. Glucose (20 g per liter) was the carbon source. Ammonium chloride (2.68 g per liter) was the nitrogen source. Phosphate (KH_2PO_4 , 0.5 g per liter and $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g per liter) was used to buffer the medium. The medium was sterilized by autoclaving

it 10 min at 15 psi and 121 C. The pH values of the experimental groups were adjusted prior to autoclaving with six N HCl or six N NaOH. The initial pH of each group was determined after autoclaving by measuring the pH of one flask in each group. The flasks were inoculated with four 0.5 cm² agar blocks cut from stock culture plates. One flask in each group was removed and its pH measured at 10 days, 14 days, 24 days, and 46 days.

To determine the optimum pH for growth of C. benekei CB-69-01 mycelium, media were prepared in accordance with results of previous experiments. The previous experiments indicated no mycelial growth occurred below a pH of 4.1, and very slight growth occurred between pH 4.1 and 4.5. Urea was used as the nitrogen source. A precipitate forms in the liquid synthetic medium containing urea if the pH values are at 8.0 or greater. The following pH values were used in the experiment: 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5. Inoculum prepared by macerating petri plate colonies produced no growth. Thus, the flasks in this experiment were inoculated with a single 0.25 cm² agar block cut from a stock culture. The initial pH of one flask in each group was checked after filter sterilizing the medium and inoculating it. Dry weights of mycelia and pH values were determined on days six and 19.

Growth curve for shake culture mycelium

To determine the growth curve for shake culture grown mycelium of C. benekei CB-69-01, eighteen flasks containing synthetic medium at an initial pH of 7.0 were prepared and each inoculated with an agar block cut from a stock culture plate. Three flasks were harvested on each of the following days: 0, 5, 12, 19, 26, and 33. The pH of the culture filtrates and the mycelial dry weights were determined.

Carbon utilization

In investigations of carbon utilization by C. benekei glucose was used as the basis for comparison for the best mycelial growth in all experiments. Each experiment consisted of several groups of five flasks containing 50 ml each of synthetic medium. The medium was prepared without carbon compounds. The carbon compounds were added separately to the appropriate groups of flasks. All compounds were added with carbon equivalent the carbon in 20 g glucose per liter. The nitrogen source was 1.5 g urea per liter, and the pH prior to filter sterilizing was adjusted to 6.0. Mycelium was harvested at 20 days, and the pH of the culture filtrates was measured. These carbon compounds were investigated: glucose, fructose, mannose, galactose, rhamnose, sorbose, inositol, trehalose,

cellobiose, maltose, sucrose, melibiose, raffinose, lactose, ribose, xylose, arabinose, dextrin, glycogen, starch, cellulose, inulin, pectin and xylan. Medium containing no carbon was also investigated.

Nitrogen utilization

Nitrogen utilization by C. benekei was investigated in a manner similar to carbon utilization. Urea was used for comparison for best mycelial growth for other nitrogen sources. The experiment consisted of 40 flasks divided into eight groups of five flasks containing 50 ml each of synthetic medium. The medium was prepared without the nitrogen compounds. The nitrogen compounds were added separately with nitrogen equivalent to the nitrogen contained in 5.06 g KNO_3 (= 0.05 M nitrogen). The carbon source was glucose at 20 g per liter. The pH prior to filter sterilizing was adjusted to 6.0 with six N HCl or six N Na OH. The mycelium was harvested and dry weight and pH determinations made at 20 days. Succinic acid (2.95 g per liter) was added to certain groups to compensate for the additional carbon provided by the deamination of asparagine. The following combinations were investigated: no nitrogen, urea, ammonium chloride, potassium nitrate, asparagine, ammonium chloride + succinate, potassium nitrate + succinate, urea + succinate.

Vitamin requirements

The investigation of vitamin requirements by C. benekei was performed in a similar manner to investigations of carbon and nitrogen utilization. The standard of comparison for best mycelial growth was the complete synthetic medium with the four vitamins: thiamine, biotin, pyridoxine, and inositol. The experiment consisted of 40 flasks divided into eight groups of five flasks each containing 50 ml synthetic medium. The medium was prepared without vitamins. The vitamins were added separately to the appropriate groups of flasks. Urea (1.5 g per liter) was the nitrogen source; glucose (20 g per liter) was the carbon source. The pH prior to filter sterilizing was adjusted to 6.0 with six N HCl or six N NaOH. Mycelium was harvested for dry weight determination at 20 days, and the final pH of the culture filtrates was measured. These vitamin combinations were investigated: four vitamins, no vitamins, biotin, thiamine, pyridoxine, inositol, biotin + thiamine, biotin + thiamine + inositol.

Optimum temperature

Stationary cultures were used to investigate the optimum temperature for growth of C. benekei mycelium. Four 0.25 cm² agar blocks cut from stock culture plates were used to inoculate flasks containing 50 ml of complete

synthetic medium. The initial pH of the medium was 6.0. Glucose and urea in the usual amounts furnished the carbon and nitrogen. Four flasks were in each group. Dry weights of the mycelium and the final pH of the culture filtrates were determined on day 20. The following temperatures were investigated: 1 C, 4 C, 9 C, 16 C, 20 C, 24 C, 28 C, 32 C and 37 C.

F. Cytology

Cytological studies were made to determine the haploid and diploid chromosome numbers, the number of nuclei in the basidiospores, the types of nuclear divisions in the basidium, and to study structural and developmental features of cells and tissues of Clitocybe benekei.

Materials for study with light microscopy were prepared as temporary or semi-permanent mounts. Although several methods were tried, no satisfactory procedures for preparing permanent slides were found. Fresh unfixed materials were mounted in lactophenol or lactophenol cotton blue for gross observation. Material was fixed in BAC fixative (Lu, 1962) or Newcomer's fixative (Newcomer, 1953).

BAC fixative contains nine parts n-butyl alcohol, six parts glacial acetic acid, and two to three parts of 10% aqueous chromic acid. This solution fixes chromosomes, nucleoli, and centrioles, produces an acid-fixation image,

and provides a good affinity for carmine stain. It was prepared fresh before use and poured over freshly cut sections of lamellae which were placed immediately into a refrigerator. Fixation takes one to five days, and the material remains suitable for use for a month or longer.

Other lamellae were placed in Newcomer's fixative, which contains by volume: six parts isopropyl alcohol, three parts propionic acid, one part petroleum ether, one part acetone, and one part dioxane. The mixture is stable and can be made up in stock solution for use when needed. Materials stored in this fixative in a refrigerator remain suitable to use for five to 11 months with either carmine or Feulgen stains.

After fixation, tissues were usually hydrolyzed in 1.0 N HCl at 60 C for 10 to 20 min depending on the condition of the material. Lower temperatures and shorter hydrolysis periods permitted better preparations of meiotic chromosomes, but did not completely clear the cytoplasm. Aceto-carmine was used for staining, and gave excellent results. Unsatisfactory results were obtained with Feulgen, HCl-Giemsa, and acridine orange staining procedures. Squash mounts of basidia from hymenial tissues were prepared. These slides were made semi-permanent by sealing the edges of the cover slips with a ring of molten sticky dental wax.

Routine observations and study were made on an American Optical (Series 10B Phasestar) microscope with built in Koehler illumination using oil immersion and dark field phase contrast. Photomicrographs were taken on Adox KB-14 film and developed with X-22 fine grain developer.

G. Toxicity and Antitumor Assays

The method described by Lucas et al. (1957) for aqueous crude extraction was used for antitumor assay. The procedure was modified by use of a buffer because unbuffered extracts had a viscous sticky consistency and did not readily pass through filters. A 1:2 (mushroom fresh weight : buffer) crude extract dilution was prepared by macerating 200 g fresh Clitocybe benekei mushrooms with 400 ml Sorensen's phosphate buffer pH 7.0 in a Waring blender. Five one min grinding periods with 30 sec settling periods between produced a thick tan foamy slurry. The slurry was filtered by gravity in a large glass funnel through four layers of cheesecloth. The residue in the cheesecloth was discarded. The filtrate was filtered through Whatman no. 1 filter paper by vacuum in a Buchner porcelain filtering apparatus. This filtrate was passed through a C-5 clarifying filter in a Seitz filtering apparatus, and finally sterilized by passing it through an ST-3 filter (pore size less than 0.1 micron) in the Seitz apparatus. During preparation the filtrates

were stored in a refrigerator and during filtering, collected over an ice bath. The sterile crude extract was frozen in 100 ml serum bottles. Dilutions of the 1:2 crude extract were made with physiological saline. The stock crude extract has a clear bright yellow color.

To determine the toxicity of the 1:2 crude extract from laboratory grown basidiocarps, albino Swiss female mice were used. Six mice in each group were injected intraperitoneally with one ml daily. Group A, the control group, received injections of sterile 0.85% NaCl in distilled water. Group B received injections of 1:2 crude extract of strain CB-69-07. Group C received injections of 1:2 crude extract of strain CB-69-04. Group D, buffer control group, received injections of Sorensen's phosphate buffer pH 7.0. Injections were given on two consecutive days, but daily observations were made for eight days.

To determine antitumor activity against Sarcoma 180 in mice, a 1:8 (mushroom fresh weight:buffer and saline) dilution of crude extract was used for the initial assay. The following procedures for testing against Sarcoma 180 in mice were used (Cancer Chemotherapy Reports No. 25; December, 1962). Six Swiss albino female mice weighing 18 to 22 g were selected for each group. Group A, as controls received one ml per day injections intraperitoneally of 0.85% NaCl in aqueous solution. Group B received one ml per day injections intraperitoneally of

1:8 crude extract of strain CB-69-04. Cut pieces of tumor three to four mm in diameter were implanted subcutaneously by a trocar in the right axillary region of the animals the day before injections began (day 0). Injections were given for seven days (day one through day seven), and tumors were cut out and weighed the day following the final injection (day eight). Animal weights were recorded on day one and day eight.

To further determine the effect of dilution on oncostatic activity, two other dilutions of the same original crude extract that was used in the initial assay were prepared. In this experiment group A received one ml per day injections of physiological saline, group B received one ml per day injections of a 1:6 (mushroom fresh weight : buffer and saline) dilution of crude extract of CB-69-04, and group C received one ml per day injections of a 1:19 (mushroom fresh weight : buffer and saline) dilution of crude extract CB-69-04. Male mice were used in this experiment.

To determine the toxicity of culture filtrates of C. benekei, six mice were given one ml per day injections intraperitoneally for two days with an undiluted 10 week old Seitz sterilized culture filtrate. This filtrate came from fungus cultures grown in liquid synthetic medium with KNO_3 in place of urea in shake cultures with a final pH of 8.8.

Two other sources besides extracts of fresh mushrooms were used to assay for the oncostatic principles of C. benekei. These were diluted culture filtrate and an aqueous extract prepared from air dried mushrooms. In this experiment group A received injections of physiological saline. Group B received an extract from dried mushrooms. Fresh mushrooms are approximately 90% water. An extract equivalent to 15% fresh mushroom weight was needed for comparing toxicity and antitumor activity with the 1:6 dilution (equals 14.3% mushroom fresh weight) of crude extract from fresh mushrooms. This was prepared from 1.5 g dried CB-69-04 mushrooms triturated to a fine powder with a mortar and pestle and added to 100 ml buffered saline solution. Buffered saline contains 25 ml Sorensen's phosphate buffer and 75 ml physiological saline. The suspension was allowed to stand over night in a refrigerator after which the pH was adjusted from 6.8 to 7.0. The extract was then clarified, filter sterilized and frozen until tested. Group C received 10 week shaker grown culture CB-69-01 filtrate diluted 1:3 (filtrate : distilled water) and the pH adjusted to 7.0. This diluted filtrate was Seitz filter sterilized and frozen until tested.

A sample of 1:6 (14.3%) crude extract from fresh CB-69-04 laboratory grown C. benekei mushrooms was sent to a laboratory of the Cancer Chemotherapy National

Screening Center for evaluation of antitumor activity against L-1210 lymphoid leukemia, and P388 lymphocytic leukemia.

RESULTS

A. Cultural Characteristics

On 1.5% malt extract agar, the outgrowth of Clitocybe benekei hyphae transferred from stock cultures isolated from basidiocarps generally cover the surface of the new plate within two weeks. Hyphae penetrate into the new agar surface and spread as a thin white cottony network across the plate, then develop a moderately thick aerial hyphal network. With age aerial hyphae become more dense and the color darkens through cream to light tan. Submerged hyphal growth also becomes more dense with age. At four weeks the reverse side of the colony is a light tan color, and a small amount of yellow pigment diffuses into the agar (Figure 7). At six weeks the aerial hyphal structure is flattened and in the hyphae on the agar surface knots of tissue begin to appear suggesting sporocarp initiation (Figure 8). These sporocarp initials are light tan colored like the vegetative hyphae, but they do not develop further in the closed petri dishes.

Cultures of C. benekei which were maintained by monthly transfer in petri plates with 1.5% malt extract agar appear stable after two years in culture. The

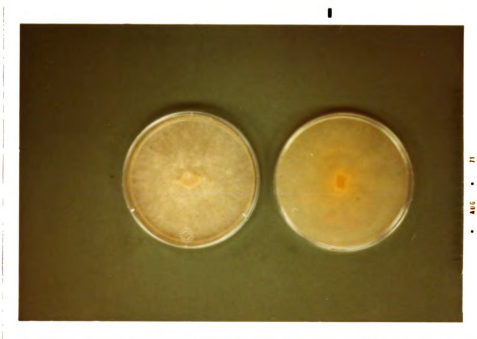


Figure 7. Four week old colony of C. benekei, top and reverse sides, on 1.5% malt extract agar.

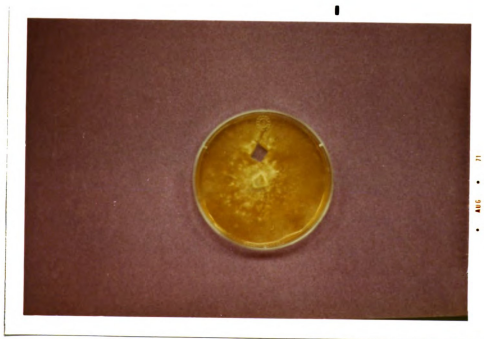


Figure 8. Six week old culture of C. benekei with tissue knots forming which suggest sporocarp initiation.

following changes in colony morphology were observed. The frequency and number of basidiocarp-like primordia developed in older colonies has decreased, and the amount of aerial hyphae produced during a month has lessened. However, colonies transferred from stock cultures and grown on a more nutritive medium such as medium A (Stevens, 1957), or 1.5% malt extract agar to which 0.5 g per liter yeast extract has been added, or the synthetic medium used in nutritional experiments solidified with agar, all developed more primordial tissue knots and more dense aerial hyphae in petri plates.

Stock cultures were maintained on 1.5% malt extract agar under sterile heavy mineral oil. Transfers made after six months from these stock cultures were all alive when inoculated onto 1.5% malt extract agar.

Basidiocarps of C. benekei have been found only on mulch on rosebeds in greenhouses, a situation where the total eradication of the organism is desired. An eradication program reported by Beneke et al. (1969) and by personal communications with the greenhouse owner is increasingly successful, and eventually may eradicate this mushroom.

B. Spore Germination

Attempts were made to germinate basidiospores of Clitocybe benekei on common laboratory media. A Sabouraud's

agar plate had a small colony visible in the spore print on day 18. During the next week numerous small white mycelial colonies developed in the spore deposits of two plates containing 4% corn meal agar, and a large mass of hyphae developed in a plate containing 2.5% malt extract agar. The latter plate was heavily contaminated with a yellow mucous bacterium, but examination of the hyphae microscopically showed the presence of large prominent clamp connections characteristic of C. benekei hyphae. By the fifth week further germinations had occurred. A second plate of Sabouraud's dextrose agar contained a small colony, new colonies developed in the plates containing 4% corn meal agar, and the first colonies appeared in plates containing Hagem-Modess medium. At this time the colony which had appeared first on Sabouraud's agar was 2.5 cm in diameter, had a thick mycelial texture, and a pale yellow-orange pigment diffusing into the agar. Eight other colonies were visible within the spore print area on this plate. When viewed through the agar at 100 X with a compound microscope, many other basidiospores were clearly seen with germ tubes emerging from them. The area of the spore print which showed the most germinating spores was the area ahead of the advancing mycelium of the original colony. The hyphae of this original colony had prominent clamp connections. After six weeks the plates of Sabouraud's agar had the greatest number of spores

germinated. The plates of 4% corn meal agar were also overgrown with hyphae, but the mycelium had a thin texture and less pigment diffusion had occurred than on Sabouraud's agar. Colonies continued to develop on Hagem-Modess medium and colonies appeared for the first time on BHI agar plates. In all plates when mycelium extended beyond the area of the central spore deposit it had developed prominent clamp connections which indicates the mycelium was that of C. benekei and not of contaminants. The dense central spore deposits in these experiments prevented the removal of recognizable single spore germlings.

In the later experiments using 1.5% malt extract agar and a beaker inverted over petri plates as a spore discharge chamber, spore germinations occurred regularly in most plates within two weeks to six weeks after "seeding."

Germination occurred mostly in plates having thicker spore deposits rather than in plates with a more scattered spore deposit, and within such plates germination occurred earliest where the spore deposit was most dense.

The spore deposits obtained from young sporocarps not fully expanded usually did not germinate. The best, earliest and most numerous spore germination occurred with deposits of spores from large fully developed basidiocarps.

When a basidiospore germinated, the hyphae rapidly grew over the medium and soon established visible colonies.

Apical hyphae of germlings were observed to proceed at a rate of five to 10 spore diameters in two hours. In the presence of an established colony, many proximal spores rapidly germinated.

C. Spawn Production

Spawn development requires four weeks for the mycelium to grow throughout the grain in the Ehrlenmeyer flasks at 24 C (Figure 9). There are no apparent differences between development on rye grain or on oats, or between flasks with CaCO_3 or without CaCO_3 . Slight differences in the rate of mycelial growth and in the density of mycelial development may be attributed to genetic variations in the stock culture strains and to the occasional presence of excess water in the bottom of the flasks. Grains of spawn removed from unused flasks after three months and placed into 1.5% malt extract agar plates rapidly gave rise to mycelial colonies indicating the organism was still viable and suitable for use at this age. When the spawn flasks were two to four months old rhizomorphs became evident and in some flasks developed profusely (Figure 10). Numerous sporocarp-like primordia but no basidiocarps also appeared on the surfaces of the grain about the same time (Figure 11). After six months spawn in flasks showed evidence of drying out, but grain placed into 1.5% malt extract agar plates produced colonies within one week (Figure 12).



Figure 9. C. benekei four week old rye grain spawn ready to inoculate in compost.



Figure 10. Rhizomorphs of C. benekei in three month old grain spawn.



Figure 11. Globose tufts of hyphae suggesting sporocarp primordial formation on four month old C. benekei grain spawn.

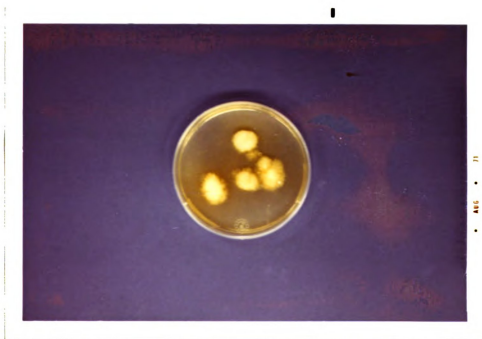


Figure 12. Colonies on 1.5% malt extract agar in one week taken from six month old spawn grains of C. benekei.

The use of cacao hulls for spawn requires enrichment to support the growth of C. benekei mycelium. The suitability of enriched Ko-K-O for spawn production is also limited due to the slower development of C. benekei mycelium on it than on rye or oats grain. Four weeks were sufficient time for C. benekei mycelium to grow completely throughout the grain in a 250 ml Ehrlenmeyer flask while over two months were necessary for equivalent mycelial development in enriched Ko-K-O substrates. However, Ko-K-O spawn is similar to grain spawn in that at four months extensive rhizomorph and sporocarp primordial formation has occurred (Figure 13). No further development of these primordia occurs after six months. Mycelial growth on enriched Ko-K-O in pint mason jars was equally slow. After six months at 24 C, only the upper two thirds of the substrate in each jar was colonized by C. benekei mycelium where rhizomorphs and sporocarp primordia formed.

D. Basidiocarp Production

The grain spawn was mixed with the compost. "Fuzzy" white hyphae appeared around the grain. After four days the hyphae grew into the compost and a small mycelial colony was established around each grain. At seven days a thin mycelial covering was present over the surface of the compost. The surface mycelium became more dense during the next 10 days and an examination of compost

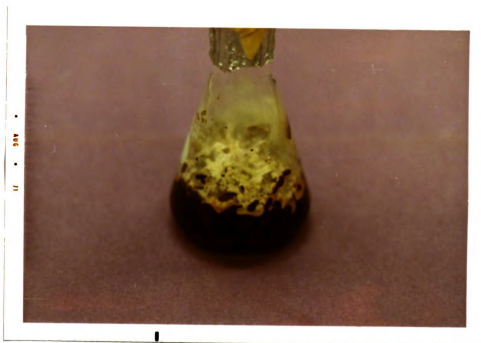


Figure 13. Rhizomorph and basidiocarp primordial formation in four month C. benekei enriched Ko-K-O spawn cultures.

showed mycelium throughout the compost in the boxes. Microscope slides of the mycelium showed clamp connections on the hyphae characteristic of Basidiomycete mycelium. Casing soil was added. Mycelium began emerging on the soil surface five days later near the edges of the boxes, but covered the soil uniformly in a few days (Figure 14). Basidiocarp primordia appeared during the second week after casing soil was added (Figure 15). The development of primordia to mature mushrooms required an additional seven to 10 days (Figures 16 and 17; Figures 18 to 21). New primordia developed as the mature basidiocarps were removed and a continuous but diminishing production continued over a two to three month period. Sporadic breaks of a few mushrooms occurred in some boxes up to four months after spawning.

Four boxes, 235, 236, 237 and 241 were spawned on July 17, 1970, while three other boxes, 253, 254 and 255 were spawned on July 31, 1970. The first group of mushrooms harvested between August 22 and December 4 yielded a range of 602 to 1003 g per square foot of compost. The second group of three boxes was harvested between September 8, 1970, and December 4, 1970. The yield was similar. For all boxes of compost with a depth of six inches, a total of 602 to 1008 g or 1.3 to 2.3 pounds of fresh mushrooms was produced per square foot (Table 4).



Figure 14. Development of C. benekei CB-69-04 basidiocarps in experimental mushroom house: mycelium emerging through casing soil.



Figure 15. Development of C. benekei CB-69-04 basidiocarps in experimental mushroom house: numerous basidiocarp primordia forming the first break.



Figure 16. Development of *C. benekei* CB-69-04 basidiocarps in experimental mushroom house: immature basidiocarps.



Figure 17. Development of *C. benekei* CB-69-04 basidiocarps in experimental mushroom house: mature mushrooms of first break, yield 550 g per sq ft.



Figure 18. Development of C. benekei CB-69-07 basidiocarps in controlled environment growth chamber: immature basidiocarps three days after primordia first appeared.



Figure 19. Development of C. benekei CB-69-07 basidiocarps in controlled environment growth chamber: immature basidiocarps five days after primordia first appeared.



Figure 20. Development of C. benekei CB-69-07 basidiocarps in controlled environment growth chamber: immature basidiocarps seven days after primordia first appeared.



Figure 21. Development of C. benekei CB-69-07 basidiocarps in controlled environment growth chamber: mature basidiocarps nine days after primordia first appeared.

Table 4. Fresh weight in grams of C. benekei mushrooms grown on compost with casing soil.

Harvest Date	Experimental Box No. ¹						
	235	236	237	241 ²	253	254	255 ³
Aug. 22				311			
Aug. 27		551	550				
Sept. 2		11		24			
Sept. 8		278		261	286	405	
Sept. 15	130		111				
Sept. 17			30				260
Sept. 25		107		95	153	137	
Oct. 5						240	170
Nov. 2	79	11		69	138	13	244
Nov. 12	402					22	132
Dec. 4	91				15	34	70
Totals:	602	1003	691	927	686	851	1008

¹Box size: 12 inches by 12 inches with 6 inches of compost.

²Boxes 235, 236, 237 and 241 spawned July 17, 1970.

³Boxes 253, 254 and 255 spawned July 31, 1970.

As evidenced by mechanical failures in both mushroom growing facilities, temporary loss of high humidity cannot be tolerated by developing basidiocarps. Loss of electricity to the humidifier in the experimental mushroom house resulted in a 20 hr period during which the relative humidity averaged 60%. A plugged valve in the controlled environment growth chamber led to the temperature rising to 92 F and a drop in relative humidity to below 70% for a similar period of time. All developing basidiocarps and existing primordia had their growth arrested at the time of the mechanical failure, and after high humidity was returned to the respective environments, no further development of these basidiocarps occurred. New primordia developed normally in these boxes during the following weeks.

In the third compost experiment the mushrooms grown at 22 C instead of 25 C in the controlled environment chamber developed on the casing soil and enlarged as usual, but as the pilei of these young basidiocarps were expanding, new primordia developed on top of them (Figure 22). Some of these epipileal primordia differentiated hymenia and discharged basidiospores. The morphology of the hymenial areas was generally of two types: (1) a globose, spongy surface covered with corrugated, short, thick lamellae which anastomosed frequently to give a poroid appearance, and (2) a typical lamellate hymenium on the

underside of a small pileus which developed out of an epipileal primordium. In the latter case this gave the appearance of mushrooms growing on top of other mushrooms (Figures 22 and 23).

Basidiocarp production without compost

1. After nearly four months from July 5 to October 26, 1969, a one inch tall basidiocarp of strain CB-69-01 developed in the aerial mycelium along the side of the glass in one of the deep culture dishes. It had a well differentiated stipe and small pileus, but no lamellae with basidia and basidiospores were formed (Figure 24). Several other tissue knots resembling basidiocarp primordia formed in the mycelium on the surface of the substrate in these culture dishes, but none developed into basidiocarps.

2. Petri plates were examined periodically for four months or longer. Although nearly all stock culture strains were grown on several media, no basidiocarps formed in any closed petri plate culture. After six weeks tufts of hyphae began appearing at the surface of the medium in most plates suggesting primordial formation. These primordia were most numerous in the more enriched media, especially media A and defined synthetic agar medium, but were present on 2.5% malt extract agar and Hagem-Modess medium. On medium A a few primordia differentiated immature brown pigmented cap-like tops.

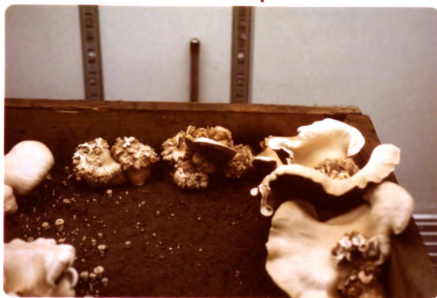


Figure 22. Sporulating hymenial areas which developed from epipileal primordia of *C. benekei* CB-69-01 at 22 C in controlled environment growth chamber.

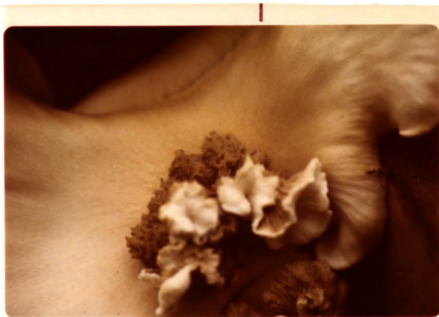


Figure 23. Corrugated to poroid and lamellate hymenial areas which developed from epipileal primordia of *C. benekei* CB-69-01 at 22 C in controlled environment chamber.

3. No basidiocarps developed in any of the cultures placed in battery jar growth chambers. Existing primordia did not develop further. New primordia-like tissue knots developed on some of the over laid sections on malt extract agar, but they developed no further than the primordia in closed petri plates.

4. After four months only a few primordia were produced in deep culture dishes containing corn meal agar. The mycelium developed very slowly and very poorly, eventually covering the surface of the medium.

5. No basidiocarps developed on 1.0% malt extract agar slants in pint mason jars, although primordia began appearing within six weeks in all cultures. The jars placed in the growth chamber developed new thickened aerial mycelium while the cultures placed on the 16 to 18 C cold air vent began drying out rapidly. One jar remaining in the constant temperature room produced a tissue knot which was one half inch in diameter, but developed no mature basidiocarp. After four months these cultures were discarded.

6. In 1.5% malt extract agar slant cultures covered on the surface with soil in pint mason jars there was no growth of the mycelium into the soil filled parts of the jars. The cultures were discarded after three weeks.

7. Nine inch by nine inch shallow aluminum foil trays with spawn inoculated soil cultures were also

nonproductive. Within three days after the cacao hulls were placed on the respective soil cultures, the cacao hulls were over grown with blue and green molds and these were discarded after a week. When examined, the soil in them had been penetrated by mycelium from the spawn inoculum grains. The trays which had been given a half an inch of casing soil soon accumulated excess water even with drainage holes in the bottom. They too were discarded when bacterial contamination destroyed the C. benekei mycelium.

8. Open deep culture dishes with various soil cased substrates were put in the experimental mushroom house for 10 days before casing soil was added. Small mushrooms of strain CB-69-03 were produced on enriched sawdust two weeks after the casing soil was added. The largest of these was one and a half inches tall with a one inch diameter cap (Figure 25). It had lamellae which discharged basidiospores. All three replicates of CB-69-03 on enriched sawdust were productive, but no primordia or basidiocarps were formed on the other substrate combinations.

9. Enriched sawdust cultures in pint mason jars did produce basidiocarps. About two months after being placed in the controlled environment chamber at 22 C, solitary basidiocarps began to appear in some of the jars with sawdust : oats (3:1) and sawdust with potato flakes

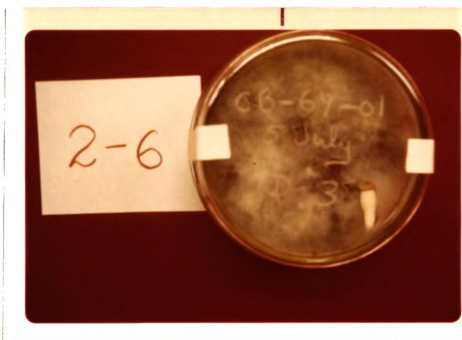


Figure 24. *C. benekei* CB-69-01 rudimentary basidiocarp in closed deep culture dish on enriched sawdust medium.



Figure 25. *C. benekei* CB-69-03 small basidiocarp on soil cased enriched sawdust medium in open deep culture dish in experimental mushroom house.

(Figure 26). They were typical of the species except that lateral or excentric stems developed in the laterally placed jars.

10. Cacao hulls in four and a half inch by eight inch by two and a half inch pans without casing soil were contaminated with blue and green molds within three days after the aluminum foil covers were removed. These pans were discarded. Cacao cultures in pans covered with casing soil were difficult to maintain with the casing soil at field capacity, and after two to three weeks excess water had accumulated in them. They were discarded also. Algae developed on the surface of the uncased grain culture pans, but after six weeks a typical mature basidiocarp of C. benekei appeared in one of these pans. It was three inches tall and had a two inch diameter pileus. Lamellae were well developed and they discharged basidiospores.

E. Nutritional Requirements

Optimum pH for mycelial growth

In order to determine the optimum pH for growth of Clitocybe benekei mycelium in shake cultures, the effect of autoclaving the defined synthetic medium using ammonium chloride as the nitrogen source was determined, and the effective pH range of phosphate buffer was established.



Figure 26. Laterally stipitate *C. benekei* CB-69-02 basidiocarp on sawdust : oats (3:1) medium in an open pint jar in controlled environment growth chamber incubated laterally.

After autoclaving the synthetic medium containing ammonium chloride the pH was lowered from 0.3 to 1.0 pH units and the color of the medium changed from clear to yellow-tan. A white precipitate formed in the flasks with initial pH values of 7.5 and 8.0. These changes in the medium due to autoclaving indicated that autoclaving was an unsatisfactory means of sterilizing the synthetic medium. After the fungus was inoculated, the pH dropped rapidly from an original range of 4.5 to 8.0 to a pH of 3.6 to 4.3 on day 14. Very little growth occurred in any flask after the pH dropped below 4.5. The use of a different nitrogen source and the need for filter sterilizing the medium was indicated.

In the determination of the optimum pH for growth of C. benekei mycelium in shake cultures in synthetic medium with urea the pH of each group was measured after filter sterilizing. Variation of the initial pH occurred during filtering of the 5.0 and 5.5 groups (Table 5), and the pH of these two groups rose more rapidly during the first six days in shake culture. This indicated phosphate buffers had little buffering capacity below pH 6.0. The optimum pH for phosphate buffers is 7.0. At day six the mycelial dry weight in all groups was nearly the same. At day 19 the mycelial dry weights ranged between 22 to 40 mg and pH values ranged from 7.7 to 8.2 (Table 5). The optimum pH for mycelial growth using synthetic medium with

Table 5. Mycelial growth and pH data of *C. benekei* using filter sterilized synthetic medium with urea and single agar block for inoculum.

pH before Filtering	pH after Filtering	Dry wt. & pH after 6 days growth (1 flask)		Dry wt. & pH after 19 days growth (3 flasks)	
		pH	grams	pH	grams
5.0	5.6	6.2	.016	7.7	.035
5.5	5.7	6.3	.016	8.0	.034
6.0	6.0	6.4	.015	8.0	.037
6.5	6.5	6.7	.015	8.1	.040
7.0	7.0	7.2	.016	8.2	.038
7.5	7.5	7.6	.015	7.7	.022

urea appears to be between pH 6.0 and pH 7.0; the greatest amount of mycelial dry weight was at pH 6.5. All culture filtrates were clear and colorless on day 19.

Growth curve for shake culture mycelium

The mycelial growth curve for C. benekei using synthetic medium with glucose, urea and an initial pH of 7.0 shows that growth increases evenly during the first 26 days when incubated at 24 C on the shaking apparatus at 150 rpm. The greatest mycelium dry weight was 53 mg on day 26. Autolysis appears to occur rapidly after the peak of the growth curve is attained as on day 33 the mycelial dry weight was 41 mg (Table 6).

Carbon utilization

A hexose, mannose, and a polysaccharide, dextrin, were the only carbon compounds among those investigated that were better utilized than glucose by mycelium of C. benekei CB-69-01 in shake culture on the synthetic medium with urea at an initial pH of 6.0. Mannose was utilized more effectively than glucose in producing mycelium when compared on a dry weight basis. Dextrin was nearly as good as mannose.

Among the other hexoses, fructose was a poor carbon source after mannose and glucose (Table 7). In decreasing

Table 6. Mycelial dry weights and pH of culture filtrates at weekly intervals of C. benekei on synthetic medium with glucose and urea at an initial pH of 7.0. Average of three cultures for dry weight.

Day	Dry wt. in mg	pH
5	21	7.3
12	29	7.9
19	41	8.2
26	53	7.9
33	41	8.3

Table 7. Hexose carbon utilization at 20 days by C. benekei mycelium in shake culture on synthetic medium with urea at an initial pH of 6.0.

Carbon Compound	pH at 20 Days	Dry wt. in Grams 20 Days
D(+)-glucose	8.3	.049
D(-)-fructose	6.9	.023
D(+)-mannose	8.0	.056
D(+)-galactose	6.2	.009
L(+)-rhamnose	7.5	.013
L(-)-sorbose	6.2	.011
i-inositol	6.3	.008
carbon free	7.0	.002

order the amounts of mycelial growth compared to glucose by all hexose carbon sources investigated were: mannose, glucose, fructose, rhamnose, sorbose, galactose, and inositol. The carbon free control had .004 g dry weight after 20 days which is probably due to the dry weight of the inoculum.

Pentoses were not good carbon sources for growth of C. benekei CB-69-01 mycelium. Ribose was the best pentose carbon source compared with glucose containing media (Table 8). The pentoses in decreasing order of utilization were: ribose, xylose, L(+)-arabinose and D(-)-arabinose.

Among the oligosaccharides tested trehalose and cellobiose gave the next best growth of C. benekei CB-69-01 mycelium when compared to D(+)-glucose in shake cultures (Table 9). The oligosaccharides are listed in decreasing order of utilization: trehalose, cellobiose, maltose, melibiose, sucrose, lactose and raffinose.

Polysaccharides as a group were more effective than the other groups of carbon sources investigated in supporting growth of C. benekei CB-69-01 mycelium in shake culture (Table 10). Dextrin was a better carbon source than glucose. Glycogen, inulin and starch were utilized by the fungus, but not as well as glucose. The exact amount of mycelium produced in cellulose medium could not be measured accurately because the finely powdered

Table 8. Pentose carbon utilization at 20 days by C. benekei mycelium in shake culture on synthetic medium with urea at an initial pH of 6.0.

Carbon Compound	pH at 20 Days	Dry wt. in Grams 20 Days
D(+)-glucose	7.8	.042
D(-)-arabinose	6.4	.008
L(+)-arabinose	6.3	.009
D(-)-ribose	7.0	.012
D(+)-xylose	6.4	.010

Table 9. Oligosaccharide carbon utilization at 20 days by C. benekei mycelium in shake culture on synthetic medium with urea at an initial pH of 6.0.

Carbon Compound	pH at 20 Days	Dry wt. in Grams 20 Days
D(+)-glucose	7.8	.042
sucrose	6.9	.011
maltose	7.2	.013
trehalose	7.2	.032
cellobiose	7.2	.023
melibiose	7.1	.013
raffinose	6.8	.006
lactose	6.4	.008
carbon free	7.0	.002

Table 10. Polysaccharide carbon utilization at 20 days by *C. benekei* mycelium in shake culture on synthetic medium with urea at an initial pH of 6.0.

Carbon Compound	pH at 20 Days	Dry wt. in Grams 20 Days
D(+)-glucose	8.3	.056
dextrin	8.2	.062
glycogen	8.2	.049
starch	7.6	.033
cellulose	7.3	.592 ¹
inulin	7.0	.038
pectin	3.5	.002
xylan	5.2	(.002) ²
carbon free	7.0	.002

¹Some powdered cellulose in mycelial pellet could not be removed.

²No growth, values estimated, inoculum blocks not recovered.

cellulose particles adhered to the mycelial pellet. At the end of 20 days a large clump of mycelium formed in the cellulose medium. In decreasing order the utilization of polysaccharides in the media compared to growth in glucose medium were: dextrin, glycogen, inulin and starch. Pectin showed no effect on mycelial growth, but this may be due to the pH of the medium falling too low after the pectin was added. Pectin is an acidic substance and the buffering capacity of the medium was probably exceeded. Xylan did not go into solution in the medium, but remained a suspension of coarse particles. No growth was recognized in the xylan medium after 20 days.

Nitrogen utilization

Asparagine was the most effective nitrogen compound for mycelial growth of C. benekei CB-69-01 mycelium in synthetic medium with glucose as the carbon source in shake culture at an initial pH of 6.0. Asparagine medium produces more mycelium than urea as the nitrogen source. Succinic acid, which is the carbon skeleton remaining after asparagine is deaminated, showed no growth promoting effect, but was retarding when supplied in equivalent molar amounts to the other nitrogen compounds in the study. The following nitrogen sources in comparison to urea for growth of mycelium are listed in decreasing order of effectiveness: asparagine, urea, ammonium chloride, urea +

succinate, ammonium chloride + succinate, potassium nitrate, potassium nitrate + succinate, and nitrogen free medium with succinate (Table 11).

Vitamin requirements

Clitocybe benekei CB-69-01, when grown in synthetic medium with glucose and urea in shake culture at an initial pH of 6.0, has no absolute vitamin requirements. A slight increase in the amount of mycelium is produced, however, when small amounts of biotin, thiamine, pyridoxine and inositol are added together in the complete synthetic medium. The differences in the amounts of mycelium produced by the addition of various vitamins separately or in combinations are very slight and may not be significant (Table 12).

Optimum temperature

The optimum temperature for mycelial growth of C. benekei CB-69-01 mycelium in stationary culture on synthetic medium with glucose and urea at an initial pH of 6.0 was 24 C (Table 13). Mycelium grown at 24 C had an average dry weight of 58 mg while mycelium grown at four degrees above or below had an average dry weight of less than half this value. No growth occurred in the flasks incubated at 37 C or at 10 C or lower.

Table 11. Nitrogen utilization at 20 days by C. benekei mycelium on synthetic medium with glucose at an initial pH of 6.0.

Nitrogen Compound	pH at 20 Days	Dry wt. in Grams 20 Days
urea	8.0	.055
ammonium chloride	4.9	.053
potassium nitrate	6.1	.028
asparagine	6.3	.067
urea + succinate	8.0	.050
ammonium chloride + succinate	6.1	.036
potassium nitrate + succinate	6.2	.015
nitrogen free + succinate	6.2	.006

Table 12. Vitamin requirements at 20 days for C. benekei mycelium in shake culture on a synthetic medium with urea and glucose at an initial pH of 6.0.

Vitamin(s)	pH at 20 Days	Dry wt. in Grams 20 Days
4 vitamins	8.0	.041
inositol	8.0	.037
pyridoxine	8.0	.035
thiamine	7.8	.033
biotin	7.9	.038
thiamine + biotin	8.0	.038
thiamine + biotin + inositol	8.0	.036
vitamin-free	7.7	.035

Table 13. Effect of temperature at 20 days on C. benekei mycelium in stationary culture on synthetic medium with glucose and urea and an initial pH of 6.0.

Temperature	pH at 20 Days	Dry wt. in Grams 20 Days
1 C	6.2	.009
4 C	6.2	.012
9 C	6.2	.010
16 C	6.8	.017
20 C	7.1	.021
24 C	7.9	.058
28 C	8.0	.027
32 C	7.4	.020
37 C	6.3	.009

F. Cytology

The complete sequence of nuclear events which occur within the basidia of Clitocybe benekei was followed from the origin of young basidia as apical hyphal cells in the hymenium through basidiosporogenesis to the formation of mature basidiospores. Characteristics of the different cells in the lamellar regions were also studied.

The cells of the lamellar trama of C. benekei are long and tubular, and individual hyphal elements are easily recognized. This kind of tissue is classified as prosenchyma and the arrangement of the hyphal elements is mostly parallel. In lamellar squash preparations individual tramal cells can be observed. Long thin unbranched cells are present in the internal regions of the trama (Figure 27). As the lamellae mature these cells elongate and their diameters increase. Tramal cells are binucleate with prominent dark staining nucleoli (Figures 27 and 28). In the nuclear area around the nucleoli dark staining heterochromatic areas can be seen. In the subhymenial areas at the periphery of the trama, cells become more irregular in shape and they are less parallel in arrangement (Figure 28), but like the internal tramal cells each end of the cell has a prominent clamp connection over the septum.

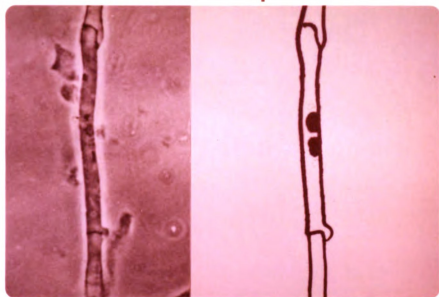


Figure 27. Internal tramal cell of C. benekei lamella. The dark spherical bodies are nucleoli; heterochromatic areas of chromosomes are present in the respective nuclear areas above and below nucleoli. 1200X.

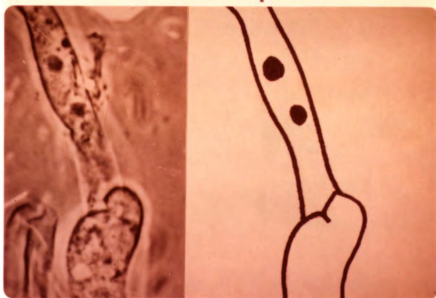


Figure 28. Subhymenial cell from C. benekei lamella with dark globose nucleoli. Some heterochromatin is present in the lighter staining nuclear area surrounding the nucleoli. 1600X.

Certain tramal cells have branches (Figure 29), and the branches most frequently originate from the anterior end of a young but fully differentiated cell. A septum with a clamp connection delimits the branch hypha from the parent cell. In this way girth may be added to enlarging lamellae as they are maturing in addition to the girth realized by enlargement of pre-existing cells.

In the hymenium ultimate apical hyphal cells differentiate into basidia, and penultimate cells characteristically form branch hyphae (Figure 30). The branch hyphae originate from the anterior ends of the penultimate cells, but not necessarily from the clamp connection which subtends the developing basidium.

The branch hypha from a penultimate cell eventually differentiates an apical basidium, usually after the formation of one or two short intervening cells (Figure 31). The new penultimate cell may again generate a branch hypha, and the capacity for differentiating more basidia and continually increasing the hymenial surface is indeterminate. Penultimate cells are very short and dikaryotic (Figure 31). All apical hyphal cells in the hymenium are potentially young basidia. No cystidia are produced by this species.

The apical cells of hymenial hyphae which differentiate into young basidia are binucleate (Figure 32). In the young basidia the two nuclei are at first positioned

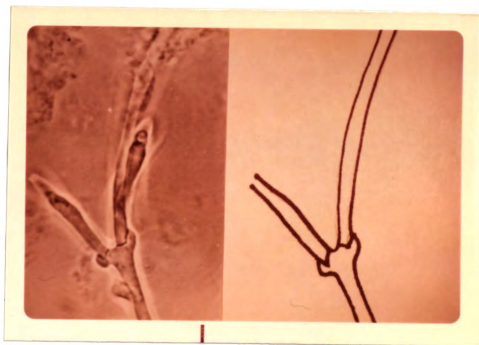


Figure 29. Branching hypha from trama of *C. benekei* with prominent clamp connections over septa. 1200X.



Figure 30. Origin of branch hypha from penultimate cell of *C. benekei*. 1400X.



Figure 31. Short dikaryotic penultimate cells of *C. benekei*. The younger basidium has differentiated from a hyphal branch of the penultimate cell of the older basidium. 1200X.

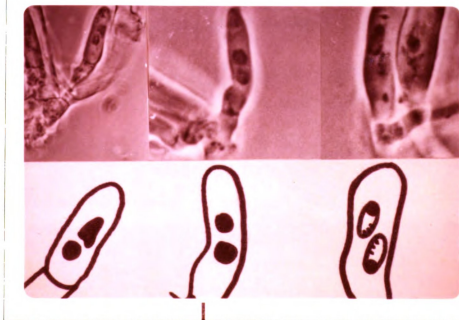


Figure 32. Dikaryotic apical cells of hymenial hyphae of *C. benekei* which are differentiating into young basidia: (a & b) very young basidia with nuclei located one above the other. (c) slightly broadened young basidium with nuclei coming into close association, nucleoli and chromomeres distinguishable within the nuclei. 1400X.

one above the other along the long axis of the cell (Figures 32a and 32b). The nuclei move into close association in the central area of the young basidium (Figure 32c). Each nucleus has a small discernable nucleolus and several dark staining granular regions in an otherwise homogeneous nucleoplasm. The nucleolus is most often seen lying against the nuclear membrane. As the nuclei come into close association in the central area of the enlarging young basidium, the dark staining regions in the nucleoplasm, the chromomeres, become more prominent.

The beginning of karyogamy apparently occurs with the breaking down of the respective nuclear membranes which allows the confluence of the contents of the two haploid nuclei. A nuclear area with two nucleoli is present for a short time (Figure 33). The chromomeres in the fusion nucleus continue to enlarge and become quite prominent. The nuclear area does not appear to be membrane bound for some time after the intermingling of the respective nuclear contents.

Meiosis begins almost before karyogamy is completed. By the time the nucleoli have fused into a single prominent nucleolus in the diploid nucleus, the chromomeres are recognized as parts of long unpaired chromosomes (Figure 34). The nucleus presents a classical leptotene appearance, but still apparently lacks a nuclear membrane

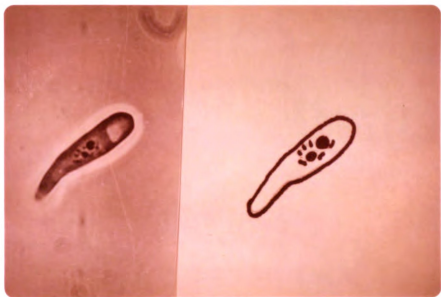


Figure 33. Karyogamy in the basidium of *C. benekei*. Two nucleoli and several dark staining chromomeres are present in the nuclear area. 1200X.



Figure 34. Leptotene in *C. benekei*. A large single nucleolus and long unpaired chromosomes are present in the nuclear area. 1200X.

(Figures 34 and 35a). The unpaired chromosomes are sufficiently distinct so that six separate strands may sometimes be counted.

In zygotene chromosomes begin synapsing (Figure 35b). They are at first very long and appear as pairs of beaded chains from the juxtapositioning of homologous chromomeric regions. The young basidium becomes uniformly more broadened during zygotene, and still apparently lacks a nuclear membrane.

During pachytene the pairing of chromosomes is completed and their chromatin condenses further. Chromosomes appear much shorter than in zygotene (Figure 36). The basidium begins elongating and the apical region is typically broader than the basal region. The nuclear area is centrally located within the basidium. The nuclear membrane appears to begin reforming around the diploid nucleus. The nucleolus is not consistently positioned within the nucleus. It is randomly located anteriorly, posteriorly, laterally or centrally, but it seems to be physically associated with a particular chromosome in many of the basidia studied (Figure 36).

During diplotene (Figures 37 and 38) the chromosomes continue to condense and they become shorter and darker staining. In weakly hydrolyzed material, the nuclear area appears to have a definite boundary which indicates the nuclear membrane is completely reconstituted

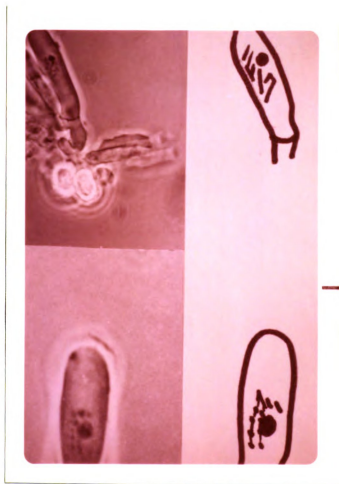


Figure 35. Meiosis in *C. benekei* basidia. (a) leptotene. (b) zygotene, long loosely condensed chromosomes with chromomeric regions juxtapositioned. a = 1200X, b = 1400X.



Figure 36. Meiosis in the basidium of C. benekei, pachytene. Partially paired chromosomes shorten and thicken, the basidium elongates and the apical end becomes broader than the basal end. 1200X.

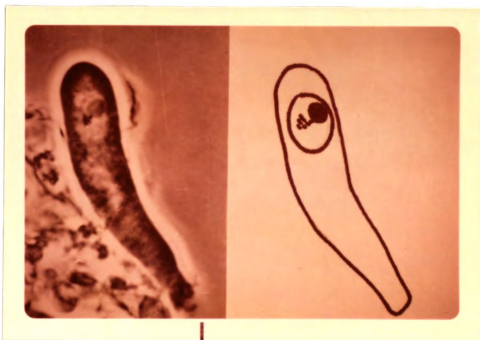


Figure 37. Meiosis in basidium of *C. benekei*, diplotene. Nuclear area has definite boundary; chromosomes shorter, thicker and darker staining. 2000X.

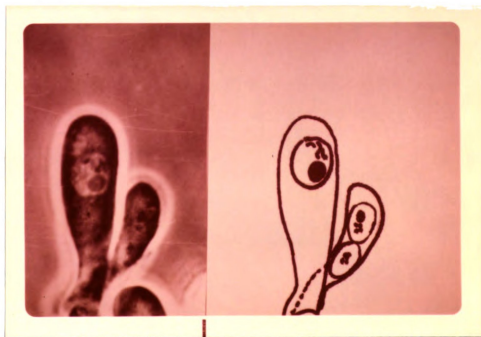


Figure 38. Meiosis in the basidium of *C. benekei*, diplotene. Definite limits to nuclear area, nuclear area located one third back from apical end of the basidium. 2000X.

around the diploid fusion nucleus by this stage. The nuclear area becomes more apically located than during pachytene where it was centrally located within the basidium. The basidium itself is undergoing rapid enlargement and elongation, and the nucleolus remains physically attached to a particular chromosome (Figure 37).

Diakinesis is the last phase of meiotic prophase I. During diakinesis the chromosomes have condensed to their shortest lengths, and bivalents are readily distinguished. Chromosome counts can be made (Figures 39 and 40). At first, three very short bivalents are seen clustered around the nucleolus, and the limits of the nuclear area are very definite which indicates the nuclear membrane does not break down during early diakinesis. The position of the nucleus is stabilized about one third of the way back from the apical end of the basidium. In late diakinesis (Figures 41 and 42) the nucleolus is no longer attached to a pair of chromosomes, and one of the pairs of chromosomes becomes longer than the other two pairs. The nucleolus is randomly located within the distinctly delimited nuclear area.

In pro-metaphase I (Figure 43a) the outline of the nuclear area is more ragged and its distinct limits are disappearing. This suggests that early metaphase is the time when the nuclear membrane disintegrates. The chromosomes are becoming more elongated and there is a clear

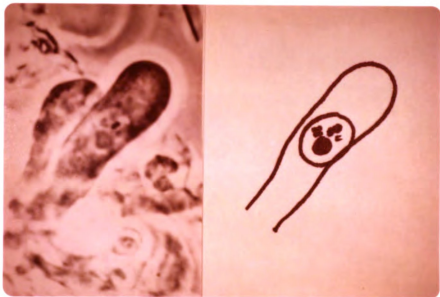


Figure 39. Meiosis in the basidium of *C. benekei*, diakinesis. Three pairs of bivalents clustered around the nucleolus. 2000X.

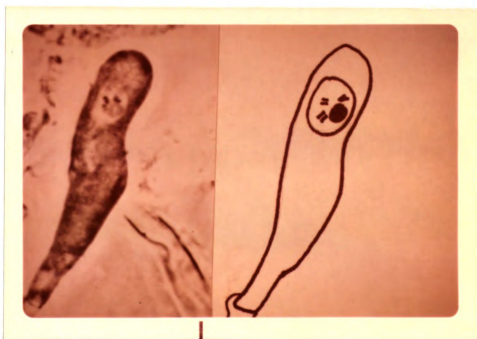


Figure 40. Meiosis in the basidium of *C. benekei*, diakinesis. Three pairs of bivalents and nucleolus in nuclear area with definite limits. 2000X.

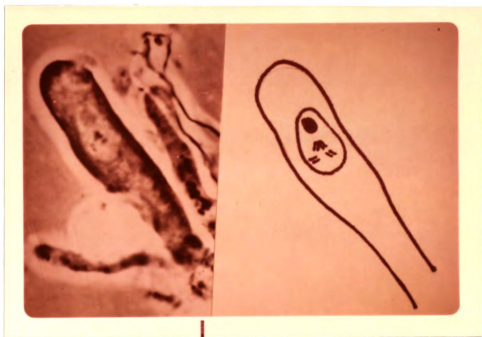


Figure 41. Meiosis in the basidium of C. benekei, late diakinesis. One pair of chromosomes is longer than the other two. 2000X.



Figure 42. Meiosis in the basidium of C. benekei, diakinesis: (a) nuclear area with definite boundaries, (b) lower level of focus showing extended length of one pair of chromosomes. 2000X.

indication of the karyotype. The long pair of chromosomes appears to have centrally located centromeres. In the two shorter pairs of chromosomes the centromeres are terminal or very nearly terminal. At a lower level of focus (Figure 43b) the nucleolus can be seen with a ragged outline that suggests it too is disintegrating during early metaphase I at the same time the nuclear membrane is disintegrating. Later in metaphase I the nucleolus and nuclear membrane are not seen. From a polar view the details of the three pairs of chromosomes lying on the equatorial plate are fairly clear (Figure 44). The nuclear area remains about one third back from the apical end of the basidium. A side view of metaphase I shows that the spindle orientation is oblique, but tends to be horizontal (Figure 45). Other cells studied at this stage indicate the spindle orientation of metaphase I in C. benekei is mostly horizontal.

Anaphase I configurations are not frequently seen. A synchronized movement of the chromosomes to the poles is suggested by the few cells studied at this stage (Figures 46a and 46b).

During telophase I the chromosomes reach the poles of the spindle apparatus and become indistinct. The loss of ability to ascertain structural detail is accompanied by a loss in staining density. Haploid daughter nuclei are located at opposite sides of the basidia. The



Figure 43. Meiosis in the basidium of *C. benekei*, pro-metaphase I. (a) Two short bivalents and one long bivalent, (b) nucleolus in same cell seen at lower level of focus. 2000X.

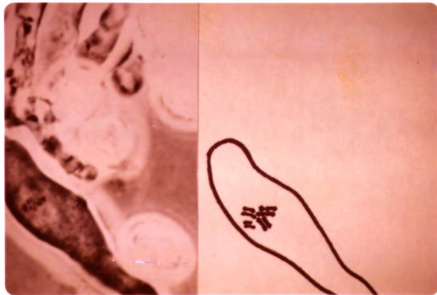


Figure 44. Meiosis in the basidium of *C. benekei*, metaphase I. Polar view of three pairs of chromosomes on equatorial plate. 2000X.



Figure 45. Meiosis in the basidium of *C. benekei*, metaphase I. Side view of chromosomes on the equatorial plate. 1200X.

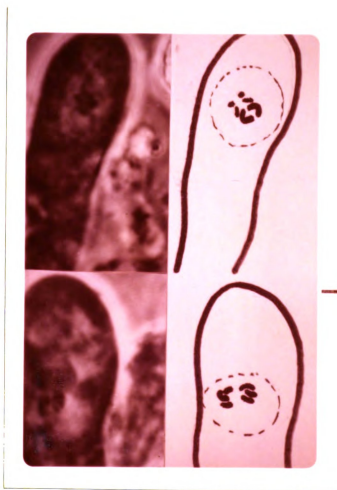


Figure 46. Meiosis in the basidium of C. benekei. Probable anaphase I configurations suggesting synchronized disjunction of homologues. 2200X.

positions of these daughter nuclei is additional evidence for a horizontal spindle orientation during meiosis I (Figure 47).

During prophase II two membrane bounded nuclei with chromomeres are present in the apical end of an enlarged basidium (Figure 48). The extreme apical location of these nuclei in this photograph probably represents an extreme variation of normal nuclear positions rather than a migration of nuclei after meiosis I. Metaphase II configurations have not been recognized in any of the material studied. Subsequent stages, however, have the nuclear apparatus located in the previously described position, one third back from the apical end of the cell.

In anaphase II univalent chromosomes are present on vertically oriented spindles (Figure 49a). The separation of daughter chromosomes seems to be synchronized.

In telophase II (Figures 49b and 50) a tetrad of four nuclei which are positioned at the poles of two parallel vertically oriented spindles is seen. Four sterigma begin to form on the apical ends of basidia during telophase II.

The post meiotic interphase nuclei do not immediately migrate into basidiospores. Instead they enlarge and migrate into a linear arrangement along the long axis of the basidium. The sterigma continue their development. Nucleoli within the nuclei are not recognizable at this

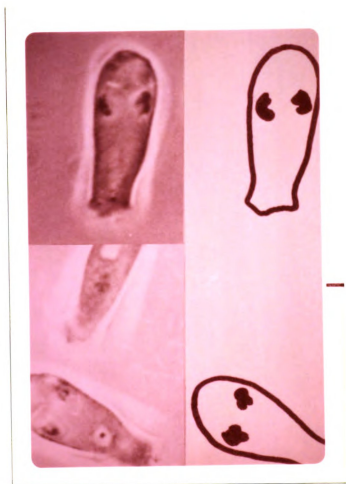


Figure 47. Meiosis in the basidium of C. benekei, telophase I. 1700X.



Figure 48. Meiosis in the basidium of C. benekei, prophase II. 2000X.



Figure 49. Meiosis in the basidium of C. benekei: (a) anaphase II, univalent chromosomes on vertically oriented spindles. (b) telophase II. a = 1700X, b = 1400X.

stage. The nuclear contents appear very homogeneous (Figures 51 and 52).

After further development of the sterigma, the nuclei migrate one behind the other toward the apical end of the basidium. When they reach the base of the sterigma they have a projecting beaked appearance on their anterior end, but have become light staining and have a diffused appearance (Figure 53).

Spores begin to bud out from the apices of the sterigma as the diffused nuclear contents move into them. The diffused nuclear material enters the developing spores through a narrow connecting channel. The spores develop asymetrically from the vertices of the sterigma (Figure 54). In the last stages of basidiosporogenesis the nuclear material becomes recognizable in the developing spores (Figure 55).

In certain mature basidiospores the nucleus is readily recognized. It has good staining affinity and is randomly located within the spore. The spore wall is thick and covered with irregular warty projections. Most spores in which the nuclear condition could be detected had a single nucleus (Figures 56a and 56b), but approximately one percent of the spores observed contained two nuclei (Figures 57a and 57b). Many spores were observed with no recognizable nuclear structure.

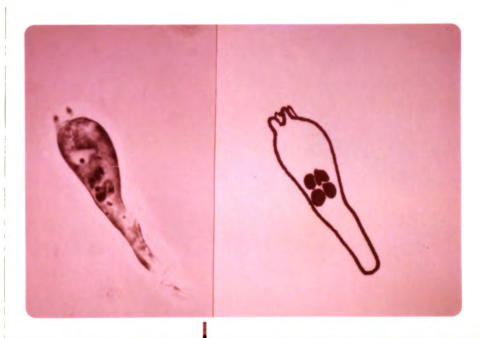


Figure 50. Meiosis in the basidium of *C. benekei*, telophase II. Four daughter nuclei forming at the poles of vertically oriented spindles. 1400X.

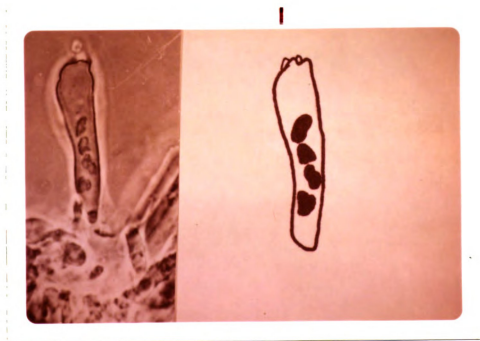


Figure 51. Post meiotic events in the basidium of *C. benekei*. Linear arrangement of nuclei following post meiotic migration. 1400X.

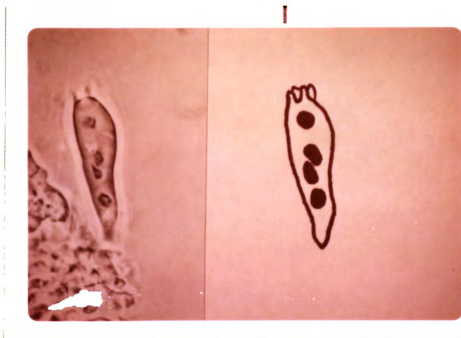


Figure 52. Post meiotic events in the basidium of C. benekei. Migration toward the developing sterigma by first nucleus from the linear arrangement. 1400X.

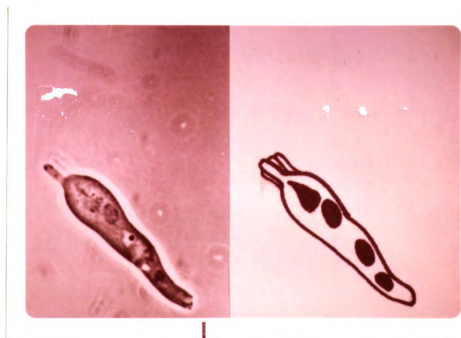


Figure 53. Post meiotic events in the basidium of C. benekei. Nucleus becoming diffused as its contents enter the sterigma. 1400X.

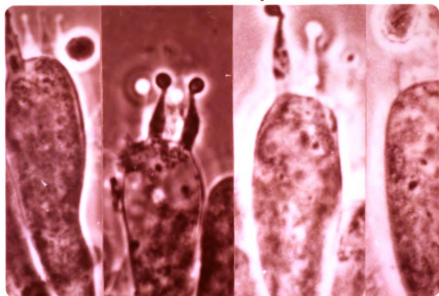


Figure 54. Basidiosporogenesis in *C. benekei*. Nuclear material enters spores through narrow sterigmatal channels in a highly diffused state. ca. 2000X.



Figure 55. Basidiosporogenesis in *C. benekei*. Dark staining nuclear matter reappearing in the developing spores. 1400X.

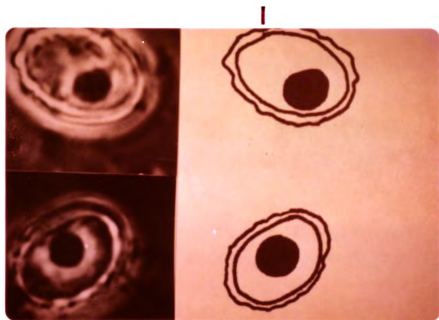


Figure 56. Mature basidiospores of *C. benekei* with one nucleus per spore. 2000X.

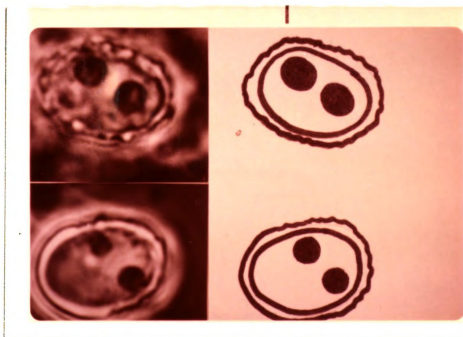


Figure 57. Mature basidiospores of *C. benekei* with two nuclei per spore. 2000X.

G. Toxicity and Assays of Crude Extracts
for Antitumor Activity

In the experiment to determine toxicity of (1:2) crude extracts from laboratory grown Clitocybe benekei basidiocarps, one ml per day injections for two days of (1:2) crude extract of strain CB-69-07 resulted in an LD₅₀ in eight days for the mice in group B (Table 14). An LD₈₄ resulted for the mice in group C which were receiving a (1:2) crude extract of laboratory grown basidiocarps of CB-69-04. No deaths occurred with the mice in either the saline or phosphate buffer control groups.

The average weight loss of the survivors on day eight in groups B and C receiving crude extracts was three g and five g per animal respectively (Table 14). During the first half hour after the animals received the first injection of the crude extract they became less active than the control animals. They walked slowly dragging their legs widely apart. Their eyes were open, but appeared cloudy and the pupils were dilated. They did not engage in the usual activities of cleaning themselves or feeding as the control groups did. On the second day before the animals received the second injection, the mice were still extremely slow in their activities and drowsy. The fur was roughened, and two of the animals receiving CB-69-04 extract had swollen bulging eyeballs. Food and water intake were consequently reduced. The animals in

Table 14. Toxicity data for 1:2 crude extract of laboratory grown basidiocarps of *C. benekei*, injected intraperitoneally one ml per day for two days into mice. Average weights in grams based on six mice.

Group	Ave. wt. day 1	Ave. wt. survivors day 8	Survivors	Survivors % animals receiving lethal dose
A physiological saline	29	29	6/6	LD ₀
B 1:2 crude extract CB-69-07	28	25	3/6	LD ₅₀ (3 days)
C 1:2 crude extract CB-69-04	29	23	1/6	LD ₈₄ (4 days)
D pH 7.0 Sorensen's phosphate buffer	28	30	6/6	LD ₀

both control groups were healthy and active. During the 24 hour period following the second injection three mice which had received CB-69-07 crude extract died. The three remaining mice in the CB-69-07 group were more active than the preceding day, but the two remaining mice in the CB-69-04 group were still quite inactive, and one died the following day. By days seven and eight all remaining mice were active and appeared normal. The crude extract of C. benekei at a dilution of 1:2 is thus too toxic for the mice as indicated by the LD₅₀ test.

A 1:8 dilution of crude extract from laboratory grown C. benekei mushrooms strain CB-69-04 was used for the initial antitumor assay. This dilution produced a 73% inhibition of Crocker mouse Sarcoma 180 in mice (Table 15). The mice which received one ml of this diluted extract became slightly drowsy shortly after receiving the daily injections, but they recovered fully before the next day. The fur was slightly roughened on day eight when compared to the control animals. Although some toxic effect is indicated, the animals in both control and experimental groups gained weight (Table 16).

In the experiment to determine the effect of dilution on oncostatic activity using 1:6 and 1:19 dilutions of CB-69-04 crude extract from laboratory grown basidiocarps, greater tumor inhibition resulted than with the initial 1:8 dilution. The mice which received 1:6 crude

Table 15. Effect of 1:8 crude extract of laboratory grown C. benekei basidiocarps, strain CB-69-04 on Sarcoma 180 in mice. Average weights in grams based on six mice.

Group	Ave. tumor wt. in mg day 8	Tumor/ control ratio	Percent tumor inhibition
A 0.85% saline	0.801		
B 1:8 crude extract CB-69-04	0.216	0.27	73

Table 16. Mice weight data during Sarcoma 180 assay with 1:8 crude extract of laboratory grown CB-69-04 C. benekei basidiocarps. Average weights in grams based on six mice.

Group	Ave. wt. mice day 1	Ave. wt. mice day 8	Ave. wt. tumor day 8	Ave. wt. mice - tumor day 8	Ave. wt. change in mice day 8 - day 1
A saline	18.8	19.8	0.8	19.0	+0.2
B 1:8 crude CB-69-04	19.8	20.2	0.2	20.0	+0.2

extract showed 90% inhibition of the tumor while those mice which received 1:19 crude extract showed 75% tumor inhibition (Table 17). All animals survived, and the fur was not noticeably roughened. After eight days the animals which received 1:19 crude extract were as active as the control mice, but in the group which received 1:6 crude extract one mouse was very drowsy and two others were noticeably less active than the control mice. All mice which received injections of crude extracts became somewhat drowsy for a short time after they received the daily injections. Only after the final seventh injection did the drowsiness remain overnight in three of the mice which received 1:6 crude extract. The maximum tolerance of crude extracts was probably reached when seven injections of one ml per day of 1:6 crude extract was given to these mice. All mice gained weight during the experiment (Table 18).

The toxicity of the filtrate at pH 8.8 from undiluted 10 week old cultures grown in synthetic medium for Basidiomycetes (glucose 20 g per liter, potassium nitrate 5.06 g per liter, KH_2PO_4 0.5 g per liter) on a rotary shaker was LD_{50} in three days. Severe drowsiness and roughening of fur occurred during the 24 hours after the first injection. After the second one ml injection was given on day two, three of the six mice died during the subsequent 24 hours. This is equivalent in toxicity to

Table 17. Inhibition of Sarcoma 180 in mice by 1:6 and 1:19 diluted crude extracts of laboratory grown CB-69-04 *C. benekei* basidiocarps. Average weights in grams based on six mice.

Group	Ave. tumor wt. in mg day 8	Tumor/ control ratio	% tumor inhibition
A 0.85% saline	0.554		
B 1:6 CB-69-04 crude extract	0.057	0.10	90
C 1:19 CB-69-04 crude extract	0.139	0.25	75

Table 18. Mice weight data during Sarcoma 180 assay with 1:6 and 1:19 crude extracts of laboratory grown CB-69-04 C. benekei basidiocarps. Average weights in grams based on six mice.

Group	Ave. wt. mice day 1	Ave. wt. mice day 8	Ave. wt. tumor day 8	Ave. wt. mice - tumor day 8	Ave. wt. change in mice day 8 - day 1
A 0.85% saline	20.2	28.5	0.6	27.9	+7.7
B 1:6 crude extract	21.3	23.8	0.1	23.7	+2.4
C 1:19 crude extract	21.0	26.3	0.1	26.2	+5.2

the sample prepared from CB-69-07 laboratory grown basidiocarps. The effect of the higher pH 8.8 of the filtrate was not investigated, but was adjusted to 7.0 for antitumor testing.

The extract from dried CB-69-04 C. benekei basidiocarps (equivalent to 15% fresh mushroom weight) produced 91% inhibition of Sarcoma 180 tumors, with some toxicity as shown by weight loss in the mice (Tables 19 and 20). The fur of these animals was slightly roughened and some noticeable drowsiness persisted between days seven and eight. The 10 week culture filtrate from a rotary shaker culture of CB-69-01 diluted 1:3 produced 73% tumor inhibition with no apparent indications of toxicity (Table 19). One mouse in this group died suddenly on the morning of day eight, but since the survivors were quite active, had no roughening of their fur, and had gained weight (Table 20) this death is probably attributed to accidental internal injury from intraperitoneal injection on day seven.

The sample of 1:6 crude extract from fresh CB-69-04 laboratory grown basidiocarps which was sent to the Cancer Chemotherapy National Screening Center was evaluated as nontoxic and inactive against L-1210 lymphoid leukemia and P388 lymphocytic leukemia.

Table 19. Inhibition of Sarcoma 180 by extracts of air dried (15% fresh weight equivalent) CB-69-04 basidiocarps and 1:3 diluted 10 week culture filtrate from CB-69-01 C. benekei. Average weights in grams based on six mice.

Group	Ave. tumor wt. in mg day 8	Tumor/ control ratio	% tumor inhibition
A 0.85% saline	0.983		
B air dried CB-69-04 basidiocarp extract	0.085	0.09	91
C 1:3 culture filtrate CB-69-01	0.267	0.27	73

Table 20. Mice weight data during Sarcoma 180 assay with dried CB-69-04 C. benekei basidiocarps and 1:3 diluted culture filtrate of CB-69-01 C. benekei. Average weights in grams based on six mice.

Group	Ave. wt. mice day 1	Ave. wt. mice day 8	Ave. wt. tumor day 8	Ave. wt. mice - tumor day 8	Ave. wt. change in mice day 8 - day 1
A 0.85% saline	19.3	19.7	1.0	18.7	-0.6
B dried CB-69-04 basidiocarp extract	17.8	14.3	0.1	14.2	-3.6
C 1:3 culture filtrate CB-69-01	20.0	20.4	0.3	20.1	+0.1

DISCUSSION

Several aspects of development were included in this investigation of the biology of Clitocybe benekei:

(A) several stock culture strains were maintained and the aspects of mycelial growth on various agar media were noted, (B) the basidiospores were germinated, (C) various types of spawn media for use in basidiocarp production were compared, (D) basidiocarps were produced under various experimental conditions, (E) the nutritional requirements for mycelial growth in shake culture on synthetic media were determined, (F) the nuclear events in the basidium and developing basidiospores were studied cytologically, and (G) extracts of basidiocarps were tested in mice for toxicity and anti-tumor properties.

A. Growth of Stock Cultures on Various Agar Media

The stock cultures which were obtained by aseptically removing pieces of pileus context tissue and placing them into 1.5% malt extract agar in petri plates at first produced numerous primordia after six weeks in culture. The decrease in primordium production after two years of monthly transfers to new plates of 1.5% malt extract agar is partially reversible as shown by increased primordium

production when transfers are made to a more nutritious medium such as medium A agar or a synthetic agar medium. This indicates nutritional requirements are important in the processes of differentiation which initiate the perfect stage. However, the great numbers of primordia which are produced in primary isolates can also be looked upon as the result of residual activity from being the immediate progeny of highly differentiated cells in mature basidiocarps. From this latter view point the involvement of endogenous regulating substances is suggested and that less of these substances is produced by mycelium in successive transfers on 1.5% malt extract agar. Gruen (1963) demonstrated that specific regions within the basidiocarps of Agaricus bisporus are the sites for synthesis of endogenous growth regulators of the basidiocarp. Bevan and Kemp (1958) noted that basidiocarps of Flammulina velutipes appeared much earlier in cultures inoculated with pieces of basidiocarp stipe than in cultures inoculated with vegetative mycelium. There was no correlation with the amount of mycelium produced and basidiocarp formation. The new primordia differentiated on the sides of the old stipe tissue. The possibility of the production of basidiocarp inducing substances in mature basidiocarps and the retention of this state of differentiation in tissue cultures, especially primary isolates, is suggested by the results of cultural studies of Xerocomus illudens (Pantidou, 1964). Two distinct types of cultures were produced when the

original isolate of X. illudens was subcultured, a fast growing mycelium which never produced primordia and a slow growing type which produced primordia after two to three weeks. When the slow growing type of mycelium is subcultured either of the two types of mycelia may appear. A possible explanation for the decrease of primordia in C. benekei on 1.5% malt extract in stock cultures and the increased frequency of primordium formation in more nutritious media is that these observations are compatible with the evidence given by other investigators for a nutritional induction of basidiocarp formation and with the evidence for autocatalytic activity from endogenous substances produced within developing or mature basidiocarps. Further unification of these observations was demonstrated by Eger (1968) with a species of Pleurotus. Extracts from basidiocarps of Pleurotus or Agaricus promoted primordium formation in Pleurotus cultures seven to 10 days earlier than in control cultures. Fractionation of the extract showed the number of primordia was increased by high molecular weight compounds, but not protein alone. A similar stimulatory effect was shown by supplying L-asparagine or urea in place of sporocarp extract for the induction of primordia of Pleurotus in culture.

B. Spore Germination

Further studies are needed to elucidate the factors which contribute to successful germination of basidiospores of Clitocybe benekei. The results of experiments reported in this thesis indicate that a period of dormancy of from two to six weeks occurs before any basidiospores germinate on agar media. The spore germination levels achieved thus far are far less than one per cent in any experiment, but definite stimulations by the mass effect of dense spore deposits or by growing C. benekei mycelium occurs in the manner Fries (1966) has reported for Agaricus bisporus. Germination appears to be better in deposits from fully mature basidiocarps than from those which have just matured and begun to discharge spores. A cytological basis for the low germination numbers may be indicated by large numbers of spores in which no nuclear structure can be detected. While most spores in which the nuclear condition could be detected were uninucleate, the binucleate condition seen in a few spores may be an indication of further maturation and a prerequisite for germination. Many agarics have typically binucleate basidiospores due to a mitotic division of the spore nucleus soon after it migrates into the basidiospore. The thick basidiospore wall may also contribute to delayed spore germination, and in some cases, may even prevent germination. In nature, external sources of cellulose and chitinase may be required by co-existence with

other organisms in the habitat. Some preliminary unreported experiments using agar overlays on the spore deposits and co-culture with Rhodotorula mucilaginosa var. sanguinea were inconclusive, but there seems to be no great stimulatory benefits from yeast co-culture. When methods for the rapid germination of large numbers of C. benekei basidiospores are found, further genetics and physiological studies with monosporous strains can be performed.

C. Spawn

Grain spawn using either rye or oats is an excellent source of inoculum for growing C. benekei mycelium for basidiocarp production. Small flasks are ready for use within a month and can be stored for six months at standard room conditions, 24 C.

Cacao hulls are not a satisfactory substrate for growing C. benekei spawn. In the "fresh" condition obtained from nursery and garden supply dealers cacao hulls do not support the growth of C. benekei mycelium without enrichment. After enrichment the growth C. benekei mycelium is very much slower than on grain. "Fresh" cacao hulls cannot serve as a casing layer due to the rapid development of imperfect and phycomycete competitors which are antagonistic to C. benekei mycelium as was demonstrated in growth chamber experiments. Personal communication with the greenhouse owner reveals that breaks of C. benekei basidiocarps do not

occur in newly established rosebeds for eight months to one and a half years. During this time the cacao is successfully composted by the activities of the other microbes and the appearance of C. benekei basidiocarps may be a stage in the ecological succession of organisms in the rosebed habitat. The significance of this is not merely to point out the unlikely choice of cacao hulls as a spawn substrate, but it also provides some contradictory information to a theory that the origin of development of C. benekei at Mt. Clemens, Michigan, was its being brought in from the tropics on the cacao. If this had occurred it would have had to have been a completely passive event with no more probability of occurrence than the entry of any other substance or organism into the greenhouse. C. benekei simply does not grow on uncomposted unenriched cacao hulls. Furthermore, spawn cultures on enriched cacao placed in growth chambers for over six months during which composting of the cacao occurred failed to produce any basidiocarps and in most cases C. benekei mycelium died out by six months even though it was firmly established on the cacao when placed in the growth chamber. This poses the question of whether cacao hulls in any manner at all play a role in the biology of C. benekei. The prominent rhizomorphs in the cacao mulch may indicate that cacao hulls are no more than a passive inert non-nutritive substance. Rhizomorphs form away from a food base and for some species may be eighty feet

or more in length (Mathew, 1961, Wilcoxson and Sudia, 1968). Mycelium of C. benekei grows profusely in the high organic soil of the rose beds, and the function of decomposed cacao is probably similar to casing soil. This being the case the total eradication of the organism at Mt. Clemens may not be achieved in the immediate future, and a continuous periodic application of fungicides such as Bordeaux or Oxine may be needed.

D. Basidiocarp Production

The formation of basidiocarps by Clitocybe benekei was achieved in the laboratory under a variety of conditions and a number of variations in basidiocarp morphology were respectively obtained. The most interesting variation was the formation of basidiocarps with epipileal primordia and the maturation of sporulating hymenial areas from the epipileal primordia. The temperature of the growth chamber was 22 F when these unusual basidiocarps formed while typical basidiocarps of this strain were produced at 25 C in the same growth chamber. Miller (1971) has attributed temperature reduction to the formation of clavarioid basidiocarps of Lentinellus cochleatus. Whether lower temperature is the only factor in the abnormal basidiocarp development of C. benekei CB-69-01 at 22 C, however, is only speculative until it can be shown that the two forms of basidiocarps are produced consistently at the respective temperatures.

Other possible causes can be suggested. Since these were not axenic cultures, the influence of other microbial organisms cannot be fully discounted. The abnormal parts of the basidiocarps were examined for the presence of other fungi, but no evidence of hyphae without clamps, conidia or conidiophores was seen even though blue and green molds did grow on the wooden boxes and on the casing soil. Algae (yellow-brown unicellular nonmotile) frequently developed on the casing soil surface, but did not grow on the basidiocarps, so there is no real evidence that the unusual forms were brought about by the activities of other microorganisms. Other possibilities are variation in the composition of compost, pH of casing soil, and age and quality of grain spawn used from one experiment to the next. While the effects of these are expected to be minimal, they cannot be eliminated as possible contributing factors to abnormal basidiocarp formation. An ancillary experiment not reported in this thesis in which two boxes of compost were covered with an inch of sawdust instead of casing soil resulted in the production of clumped mostly sterile carpophoroids after four to five months. This occurred at 22 C in the experimental mushroom house. Since sawdust holds water very well the mycelium developed a thick matted texture on the surface with liquid accumulation below prior to carpophoroid formation.

If temperature alone were responsible for the abnormal basidiocarp development in the growth chamber then no normal basidiocarps should have developed in the experimental mushroom house unless the effect of temperature can be modified by other factors. The interacting of temperature and relative humidity could be important to basidiocarp formation. A suggested basis is that 22 C and 80 to 90% relative humidity gives normal basidiocarps in the experimental mushroom house; 25 C and 90 to 95% relative humidity gives normal basidiocarps in controlled environment chamber; 22 C and 95+% relative humidity in the controlled environment chamber gives basidiocarps with epipileal primordia.

In the experimental mushroom house at 22 C at a relative humidity of 80 to 90% certain mushrooms on compost with casing soil had slightly scaly pilei which were identical in appearance to the basidiocarps in the controlled environment chamber at 22 C at 95% relative humidity when the epipileal primordia began to appear. Certain other mushroom clusters developed corrugated ridged anastomosing hymenial areas in the axils of a cluster of stipes. This indicates the evidence of lower temperature on abnormal fruit body formation did occur in the experimental mushroom house, but it was modified by a lower relative humidity.

The cause of abnormal basidiocarp formation may be due to alteration of gene activity. Watling in studies of

polymorphism in Psilocybe merdaria (1971) has demonstrated the spontaneous development of teratological forms, and he cites several reports in the literature to demonstrate that the development of abnormal fruiting structures is not completely uncommon among higher Basidiomycete species. But, in a final analysis it seems that temperature is likely to be an important contributing factor for the abnormal basidiocarps produced at 22 C in the controlled environment chamber.

Relative humidity is very important to the development of C. benekei basidiocarps. During the first weeks of mushroom production in the experimental growth chamber, relative humidity was maintained between 80 and 90% and basidiocarps with fully expanded symmetrical pilei formed. In the following weeks relative humidity was maintained at only 74 to 84% averaging about 80%. Basidiocarps continued to form, but grew slower and some pilei became asymmetrical. At one point when electricity to the humidifier was shut off for 20 hours the relative humidity dropped between 60 and 70% and all basidiocarps and primordia had their development arrested at the point prior to the loss of higher relative humidity. Primordia did not develop further after relative humidity was returned to between 80 and 90%. A relative humidity of 80% seems to be the minimum for normal basidiocarp development. The relative humidity in the controlled environment chamber was maintained at 95%.

Relative humidity and temperature may serve as modifying influences of basidiocarp formation by their interaction.

The moisture level of the casing substance is quite critical (Atkins, 1966). When too dry no primordia form, when too moist, water forms a surface tension film on which a tight mycelial mat forms. Arbuscular structures developed from cultures in the growth chamber when this occurred and clumps of mostly sterile carpophoroids developed on a surface film of water on sawdust in the experimental mushroom house.

Accumulated metabolic CO_2 was probably a contributing factor to the failure of development of C. benekei primordia in closed petri dishes and deep culture dishes. The inhibitory effects of CO_2 on basidiocarp initiation and development are well known. Aeration is necessary for fruiting of Flammula alnicola in culture, but not for F. conissans (Denyer, 1960). However, larger basidiocarps of F. conissans are produced if cultures are aerated after primordia form. San Antonio (1971) found that aeration during the time of mycelial growth on grain was important to subsequent initiation of fruiting of Agaricus bisporus in addition to the aeration after casing soil is applied. Niederpruem (1962) found fruiting of Schizophyllum commune restricted in sealed petri plates. Plunkett (1956) and Long (1962) found pileus expansion of Flammulina velutipes was inhibited by CO_2 accumulation. A similar inhibition of basidiocarp

formation by Polyporus palustris was noted by Flexer (1963). Mader (1943) reported the need for aeration for Agaricus campestris development, and Lambert (1933) demonstrated CO₂ reduced the size of basidiocarps of A. campestris. Escobar (1970) reported that the CO₂ concentration under sterile casing soil with A. bisporus was maintained above 0.1% during the entire growing period. He noted that air flow of 1000 ml per min in experimental containers was best for mushroom production. At this flow rate CO₂ concentration above the casing soil decreased to less than 0.02% at the time of primordial formation. This agrees with reports of Long and Jacobs (1969) that a small amount of CO₂ is necessary for basidiocarp initiation, and the results of Denyer (1960) that Flammula conissans requires a still atmosphere for primordium initiation, then aeration for best basidiocarp development.

Clitocybe benekei basidiocarp production was highest when casing soil was added indicating its application was a factor in initiation of primordial formation. Escobar (1970) agrees with Tschierpe (1959) that casing soil creates a carbon dioxide gradient between the air in the compost and air above the casing soil and that basidiocarp formation by A. bisporus occurs in a region with a partial CO₂ pressure gradient. Escobar further hypothesizes that the activity of microbes in casing soil which other investigators thought to provide the stimulatory function of casing soil

may be explained by the evolution of CO₂ by these other microorganisms. Escobar was unable to demonstrate a role for ethylene in the metabolism of A. bisporus.

Whether aeration should be discussed as a separate factor from the inhibitory effects of CO₂ is questionable since the greatest value in providing aeration to developing basidiocarps is in the removal of accumulated metabolic CO₂. Yet, the replenishment of oxygen, dispersion of volatile organic metabolic gases and increased rate of transpiration have been suggested as ancillary functions of aeration by some investigators.

Numerous factors effecting basidiocarp formation by Hymenomycetes and Gasteromycetes have been determined, but some of these are better understood than others. Presumably there is a critical amount of vegetative mycelium which needs to develop before fructifications can be formed. The amount is sometimes very small. McLaughlin (1964) produced fruit bodies of Suillus rubinellus on a sparse mycelium consisting of only the inoculum block and a few millimeters of hyphae surrounding it. Other species that readily form basidiocarps from the mycelial mass within a petri plate are: Flammulina velutipes, Pleurotus ostreatus, Schizophyllum commune and small species of Coprinus. The primordium-like tissue knots formed by strains of C. benekei in petri plates indicate that the size of the mycelial mass is not necessarily limiting the development of fruiting in

these dishes. With certain culture modifications Bigelow (1970) obtained fruit bodies of C. tenuissima on malt extract agar in petri plates.

The autocatalytic effects of basidiocarp inducing principles produced in developing and mature basidiocarps have been discussed above in relation to primordium formation in stock cultures in petri plates.

Nutrition as a factor in basidiocarp formation was suggested by increased primordium formation of C. benekei on the more enriched media. Eger (1968) demonstrated stimulation of primordium formation in Pleurotus sp. by urea and L-asparagine. The failure of basidiocarps to form in the growth chambers on enriched cacao spawn media and on soil-cased enriched cacao in the experimental mushroom house is interpreted at least in part to nutritional deficiency of the cacao substrate in contrast to the compost substrate. A summary of studies of nutritional regulation of basidiocarp formation on synthetic media was recently published by Volz and Beneke (1969).

The genetics of a higher Basidiomycete mycelium often determine its capacity for basidiocarp formation. The mere fact that a stock culture was obtained from a genetically compatible and genetically complete mycelium in a basidiocarp by tissue culture does not guarantee this stock culture is genetically complete for basidiocarp formation. In the discussion following Miller's (1971) paper,

Dr. Nobles points out the common occurrence of reversion of parts of dicaryotic polypore cultures to the haploid condition. With Trametes americanum the whole mycelial mat reverts to haploid. In C. benekei stock cultures the morphology of the colonies and the regular appearance of clamp connections on the mycelium has remained constant although the frequency of primordium formation on 1.5% malt extract agar has decreased. This may be a nutritional factor as modification in the medium results in an increase in primordium formation. A loss in the genetic potential for basidiocarp formation in the stock cultures would have to be investigated further.

Light is known to affect basidiocarp development in several species. Bigelow (1970) found light necessary for basidiocarp formation in C. tenuissima. In the studies of C. benekei continuous lighting was supplied, but the exact nature of the light requirement was not determined. However, developing stipes in wooden boxes grew toward the light source and lateral stipes of basidiocarps grown in jars placed horizontally were positively phototropic. Banbury (1959) reports positive phototropism by species of Lentinus, Coprinus and Collybia, and that Psalliota campestris shows no sign of phototropism.

The pH optimum for fruiting has been investigated for a few higher Basidiomycete species. Values from 5.0 to 7.2 are reported indicating an acid pH is important

rather than alkaline (Volz and Beneke, 1969). A preliminary investigation of the pH beneath clumps of fruit bodies of C. benekei in the rosebeds at Mt. Clemens showed that the pH in the upper layers of partially decayed cacao bean hulls mulch averaged 5.3, but the pH of the soil beneath the mulch averaged 5.9. The pH of casing soil used in experiments in the experimental mushroom house for production of C. benekei basidiocarps was 6.0. The optimum pH for shaker grown mycelium of C. benekei was 6.5. An acid pH is apparently optimum for both vegetative and reproductive growth of C. benekei.

In conclusion, the formation of basidiocarps by a Homobasidiomycete organism whether Hymenomycete or Gasteromycete requires a delicate balance among many factors some of which are inherent while the others are environmental. Environmental factors are both physical and chemical, and their requirements by the organism are not merely qualitative, but quantitative as well. The conditions bringing about primordium initiation may sometimes inhibit maturation or at other times favor development of the fully formed basidiocarp of C. benekei.

E. Nutritional Requirements of Clitocybe benekei Mycelium

The optimum pH for growth of C. benekei CB-69-01 mycelium in shake culture on synthetic medium is 6.5 for a 20 day growth period at which time a maximum rate of growth occurred. Mannose and dextrin were better carbon sources than glucose, and asparagine was a better nitrogen source than urea. No vitamin requirements were demonstrated. A slight stimulatory effect on growth occurred when the four vitamins were included in the medium.

For maximum mycelial growth of C. benekei, synthetic medium should be prepared with dextrin as the carbon source, L-asparagine as the nitrogen source with trace elements included. When phosphate buffer is added (at 1.0 g KH_2PO_4 and 0.5 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$), the resultant pH has been 6.3. This is also the final pH after 20 days growth in asparagine medium, and is very near the 6.5 optimum initial pH indicated in experiments. Increased yields are expected at 20 days on this slightly modified synthetic medium containing dextrin and asparagine with an initial pH of 6.3.

An extensive discussion of nutritional requirements for mycelia of higher Basidiomycete species has been published by Volz and Beneke (1969).

F. Cytology

Meiosis in the basidium of C. benekei appears typical for eucaryotic organisms. No centrioles were seen although there was evidence of spindle fibers. Studies by

Lu (1967b) with Coprinus lagopus show that centrioles and spindle fibers are regularly present. Future modification of technique may show centrioles in C. benekei. The status of centromeres was only indicated by a thin-staining sometimes bent region of the long pair of chromosomes. Studies involving electron microscopy are needed to show the actual manner of spindle fiber attachment to chromosomes as well as give a more detailed picture of the structures of spindle fibers, synaptonemal complexes or centrioles.

Of interest is the recording of post meiotic nuclear migrations into a linear arrangement. This same pattern of migration was observed by Ehrlich and McDonough (1949) in Schizophyllum commune. Other nuclear events of C. benekei are similar to those reported for S. commune, however, no box-like arrangement of chromosomes was observed in diakinesis. In C. benekei the spindle orientation of the second meiotic division seems to be at right angles to the first, both are transverse in S. commune. The loss of nuclear membranes before chromosomes migrate into developing basidiospores is a common occurrence in both species, but the actual passage of stained recognizable chromosomes into developing basidiospores was not seen in C. benekei. The chromosomes of C. benekei may be in a less condensed state than S. commune, but like S. commune may also pass through the narrow channel of the sterigma one at a time. The

nuclear condition of basidiospores when discharged is typically two per spore for S. commune but mostly one per spore for C. benekei.

Post meiotic events in Boletus versipellis and other boletes differ from C. benekei and S. commune (Duncan, 1970). In meiosis II four nuclei remain in the upper part of the nucleus, and in the interphase following meiosis II enlarge and develop prominent nucleoli. The nuclei become beaked and advance toward the sterigma. The chromatin becomes extremely condensed and attenuated as it progresses into the basidiospore, but the nucleolus becomes detached from the chromatin and returns through the sterigma to the basidium. The nuclei in the spores divide mitotically and one daughter nucleus in each spore migrates back into the basidium. The nuclear condition of basidiospores of boletes is one per spore at the time of discharge.

Wilson, Miller and Griffin (1967) reported the nuclear behavior in the basidium of Fomes annosus. Some variations occur which were not seen in C. benekei. Centrioles are readily seen and prefusion nuclei appear double stranded. Nuclei fuse laterally and the fusion nucleus at first appears four stranded with prominent heterochromatic regions on the strands. This is difficult to relate on a chromosomal basis as the chromosome number is subsequently indicated as seven pairs in diakinesis. Sterigmata appear during pachytene, and spindle orientation

during meiosis I is vertical. The nucleolus persists through anaphase I. The disjunction of homologues is non-synchronized during anaphase I and II. The mention of paired chromosomes at metaphase II is confusing. After the nuclei enter the spores a mitiotic division occurs and spores are typically binucleate. These authors insist their findings are similar to those of others who have studied meiosis in higher fungi, but fail to cite any similar related studies. The implication of centrioles as organelles of locomotion for nuclear migrations is worthy of note and seems very probable.

The study by Huffman (1968, 1969) of meiosis in Collybia maculata var. scorzonerea reveals chromosomes elongate prior to pachytene. The haploid chromosome number is eight and centromeres are distinguishable on the three longest pairs of chromosomes. After meiosis the nuclei migrate basipetally and then apically into the sterigmata.

Chang and Chu (1969) reported meiosis in Volvariella volvacea occurs within the confining nuclear membrane of the original fusion nucleus. In Coprinus lagopus (Lu, 1967) the nuclear membrane disappears during metaphase I, as was observed with C. benekei.

G. Antitumor and Toxicity

Present indications are that basidiocarps of Clitocybe benekei are inedible. Aqueous extracts injected

into mice were lethal when undiluted and other side reactions were evident in some diluted samples. Personal communication of Dr. K. Cochran of the department of Public Health at the University of Michigan to Dr. Beneke indicated sporocarps collected at Mt. Clemens were toxic when immature but certain mature basidiocarps were nontoxic.

Over 90% reduction of Sarcoma 180 tumors in mice can be achieved with extracts of C. benekei basidiocarps. In certain animals complete regression of the tumor occurs although a toxic reaction appears.

Results of tests by CCNSC of a 1:6 diluted sample of basidiocarp extract showed inactivity against two forms of Leukemia.

SUMMARY

The development of Clitocybe benekei was studied with respect to the growth of mycelium, the germination of basidiospores, spawn media, the production of basidiocarps, the nutritional requirements, and the nuclear events occurring in basidia and developing basidiospores. Toxicity and antitumor properties of crude extracts were evaluated.

Stock cultures appear stable after two years in 1.5% malt extract agar. The formation of primordia in colonies developed from inocula taken from stock cultures were influenced by modification in the nutrients in the medium. However, endogenous growth regulators in primary isolates, which are the progeny of tissue of highly differentiated reproductive organs, apparently influenced primordial formation.

Basidiospores germinated on several agar media after a two to six week incubation period at 24 C. Many spores studied cytologically had no discernable nuclei which may account for the low per cent germination. Less than one per cent of the spores observed had two nuclei.

Grain spawn developed in a month and remained viable for six months. Cacao spawn is unsatisfactory because it required enrichment and developed very slowly.

Basidiocarps can be produced under controlled conditions in the laboratory. Typical basidiocarps developed on horse manure compost with casing soil in growth chambers. Continuous light and aeration were used with a temperature of 25 C and 80 to 95% relative humidity. At 22 C abnormal epipileate forms were produced with lamellae or poroid surfaces on the basidiocarps.

The optimum pH for mycelial growth in shake culture on synthetic medium is 6.5. Mannose and dextrin were better than glucose as carbon sources. Asparagine is a superior nitrogen source to urea or ammonium chloride. No vitamins are required. The optimum temperature for vegetative growth is 24 C.

The basidial cytology of C. benekei is similar to other Hymenomycetes which have been extensively investigated, but has several distinct features. The haploid chromosome number is three. A post meiotic migration of the nuclei into a linear arrangement precedes migration of the nuclei into the basidiospores. Mature basidiospores are typically uninucleate.

Undiluted basidiocarp extracts are highly toxic when injected into mice. Extracts possess significant activity against Crocker mouse Sarcoma 180 in mice. No activity was demonstrated against two forms of Leukemia in mice.

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