THE BIOLOGY OF *TREMELLA BAMBUSINA* SACC.

by

SHERMAN G. BROUGH
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required standard.

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Department of Botany

The University of British Columbia
Vancouver 8, Canada

Date 24 June 1970

ABSTRACT

This study was carried out on the Heterobasidiomycete *Tremella bambusina* Sacc., a tuberculate jelly-fungus growing from perithecia of species of the pyrenomycete genera *Valsa* and *Diaporthe*.

Single spore isolates of basidiocarps from a range of hosts were crossed and the compatibility system was found to be of a modified tetrapolar type. Two "A" alleles needed for initiation of conjugation tubes and 13 factors of the "B" locus determining dikaryotization, were identified from isolates of seven basidiocarps.

Culture studies were carried out on the haploid yeast-like phase in liquid media and growth was measured by optical density. Rapid growth was obtained in chemically defined media when glucose, sucrose or maltose were used as carbon sources, where ammonium compounds were used as nitrogen sources, and where the vitamin thiamine was added to the synthetic medium. Good growth occurred at pH levels from 4.0 to 7.0 with optimum growth near pH 7.0. Most rapid growth took place at temperatures between 15° and 20° C. At higher temperatures (room temperature) rapid growth occurred when calcium compounds were added. Sodium acetate as a carbon source and the nitrate ion as a nitrogen source did not provide for good growth.

Conjugation hormones were measured to be effective over a lateral range of 20 mm. in agar medium. These hormones behaved similarly to those of species of *Tremella* investigat-

ed by other workers, except that no tube initiation could be established after the hormone was autoclaved. In liquid media, relatively few conjugation tubes occurred and conjugation could not be obtained.

Initiation and formation of conjugation tubes as well as growth of the dikaryotic phase was inhibited by diffusible substances produced by the haploid phase.

Conjugation tubes, of compatible types, growing on agar medium, were tropic to each other within a 10 μ. range. Observations on conjugation and subsequent dikaryotic growth were recorded.

Clamps with haustoria as well as simple clamps were common at specific stages in development. Exposed mycelium on a variety of media, developed into basidiocarps within 10 days. No reaction with *Valsa* was observed in culture.

Culture conditions affect the texture, structure and size of the basidiocarps, the hymenial surface, and basidial production.

The life cycle was completed in culture, but basidiospore production was erratic. Conditions affecting basidiospore germination by budding, by repetition on the hymenial surfaces, and tube formation in the presence of conjugation hormones, were examined.

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INTRODUCTION

I. Objectives of study

The genus *Tremella* is abundantly represented in the mycobiota of the moist Pacific Coastal region of Canada. The majority of *Tremella* species recorded here are found on species of conifers and woody angiosperms, already infected by other fungi. This habit, being typical of the genus, limits its economic importance and has attracted little attention. The genus is cosmopolitan and few studies have been made on this group other than enumerations and descriptions of common forms. No world monographic study has been published although several regional monographs have appeared (Gilbert, 1910; Neuhoff, 1931; Martin, 1952; McNabb, 1964). Three comprehensive cytological investigations (Neuhoff, 1924; Looney, 1933; Whelden, 1934) and investigations of sporé germination and cultural studies by Brefeld, (1888) and Möller(1895) have been reported. More recently examinations of *T. mesenterica* Fr. concerning aspects of conjugation (Bandoni, 1963, 1965) and identification of some extracellular polysaccharides (Slodki et al., 1966; Slodki, 1966) were published.

Among the commonly collected species of *Tremella* from this region are several small, variable, tuberculate forms found on perithecia of Ascomycetes. These forms are associated with a variety of angiosperm and gymnosperm species and are encountered frequently during wet weather. In

the identification of *Tremella* species, some emphasis is placed upon the color, texture, shape and size of basidiocarps, as well as substrate or host fungi. The size and structures of various microscopic features such as spores, basidia, conidia and the presence or absence of clamps also has been ascribed as important. There is an almost continuous availability of local tuberculate forms, showing variation of many of these features. Spores of these forms can easily be obtained, germinated and the fungus maintained in culture. It seemed appropriate to determine, under controlled conditions, the role of hereditary and environmental factors upon these features, and in so doing, provide information that may give greater confidence in species delimitation.

A study of the role of nutrition, temperature, pH, and other environmental factors upon the growth and development of *Tremella* is partially simplified by the inherent advantages of culturing the haploid yeast-like stage in liquid media. In addition, a convenient and quick approximation of growth, as well as standardization of inoculum amounts using turbidimetric measurements, can be made.

Basidiocarps of *Tremella* are irregular and of variable form and size. They have amphigenous hymenia and lack differentiation into stipe and pileus characteristic of most Basidiomycetes used in developmental studies. The development of these structures is often reported to be influenced

by many different and complex factors. The conditions influencing growth and fruiting of *Tremella* would appear to be less complex and more easily studied.

A study of some physiological requirements was made with the following objectives in mind: 1. To find media providing for a rapid and normal growth of the various phases of the organism. 2. To produce good growth on chemically defined medium so fundamental for future work in developmental and sexual studies. 3. To induce the most rapid and consistent conjugation, and growth of normal basidiocarps, basidia and basidiospores, and 4. To observe effects of specific factors upon the development of the fungus in a controlled environment.

The use of *Tremella* in Basidiomycete sexual studies, could simplify some of the inherent drawbacks involved with many of the forms previously employed. The easily distinguished dimorphic growth phases characteristic of the genus, represent a distinct advantage over other frequently-used basidiomycetes possessing mycelial characteristics in both the monokaryotic and dikaryotic stage. Dedikaryotization is readily recognized without the use of special techniques and in fact can be ascertained macroscopically. The techniques of replicate plating can be applied in studying the morphologically distinct and easily maintained yeast-like haploid phase. The limited conjugation tubes and simple morphogenic changes associated with conjugation, reduce the problems

normally associated in determining compatibility. In addition, a sexually regulated hormonal system controlling conjugation (Bandoni, 1965) as well as an unique tetrapolar sexual pattern reported for *T. mesenterica* (Bandoni, 1963) created interest in an examination and comparison of these aspects in the tuberculate *Tremella* species.

II. The genus *Tremella*

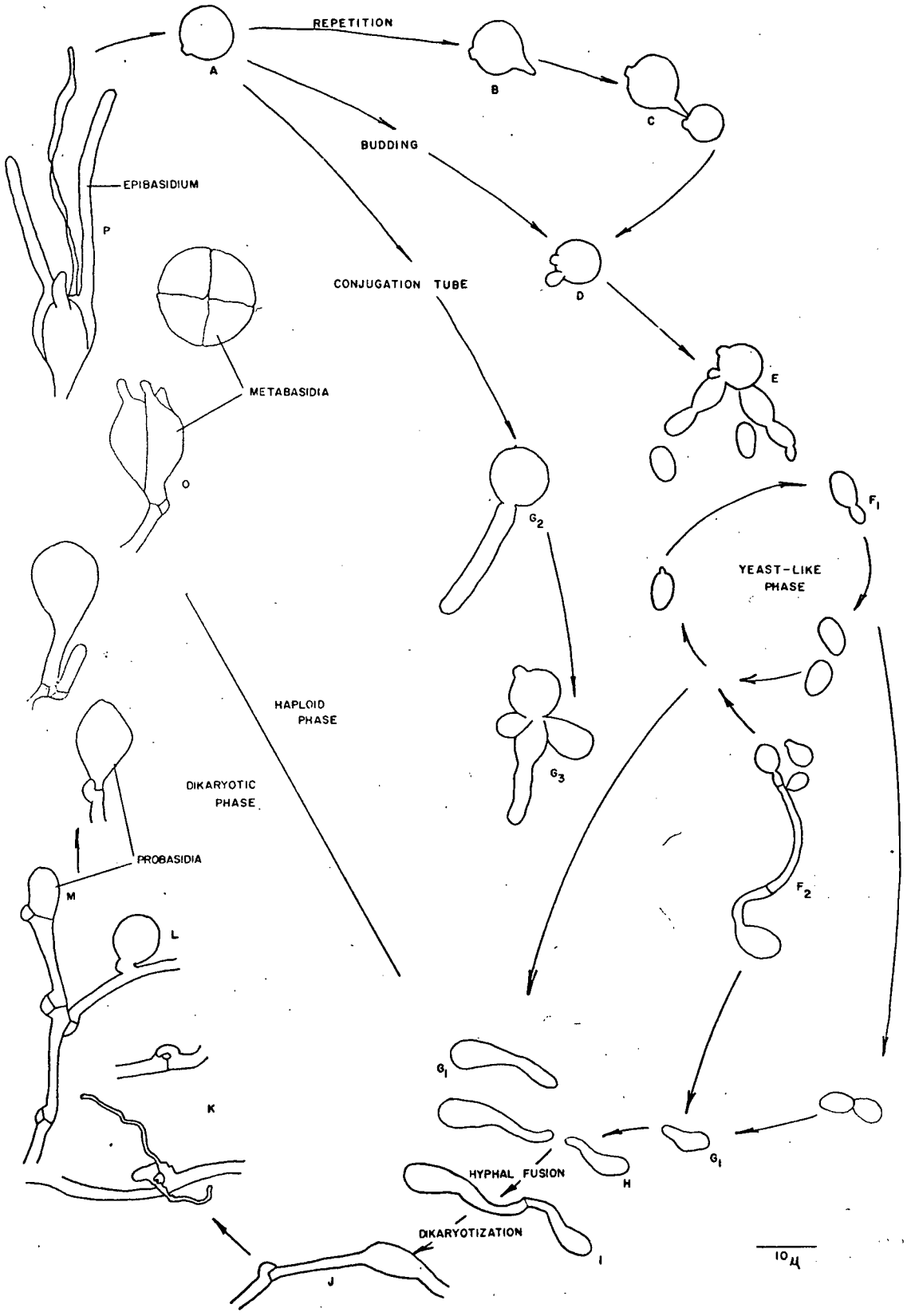
An excellent historical review of the name *Tremella* is presented by Donk (1958) who discusses the development of the *Tremella* concept. Dillenius (1741) first used the name *Tremella* to represent what is now mainly a group of algae (*Nostoc*); later Linnaeus (1753) included as well, some species now placed within the groups Fungi Imperfecti, Lichens and Basidiomycetes. Since the early 19th. century, there has been a series of attempts to bring a semblance of order to the genus. A gradual restriction of the taxon to an increasingly homogeneous but poorly known group of fungi within the order Tremellales has been accomplished. *Tremella* is placed in the order Tremellales, which together with the Uredinales (Rusts) and the Ustilaginales (Smuts) are now frequently grouped together under the form subclass Heterobasidiomycetidae (Ainsworth, 1967; Alexopoulos, 1966; Lowy, 1968).

Looney (1933) presents a historical review of the literature up to the early 1930's, of the genus *Tremella*. As in many other groups of fungi, investigations up until 1900 were mainly taxonomic in nature and involved mostly the gross morphology of the basidiocarp.

Early notable exceptions to the short, macroscopic descriptions were those of Corda (1837) who first illustrated microstructure of *Tremella* (*Naematelia*) basidia, and Tulasne (1853) who also made accurate observations of basidia and germinating basidiospores of two species of *Tremella*. Brefeld (1888) and Möller (1895) studied *Tremella* species and clearly illustrated germinating basidiospores in water and nutrient media, showing budding and the production of conjugation tubes.

Despite the early attempts at culturing *Tremella*, cytological studies seemed to be of prime interest from 1900 to the 1930's. Whelden (1934) gives a brief review of the studies of Istvanffi, Dangeard, Marie, Neuhoff, and Kuhner. These works concentrated mainly upon the formation of clamps, basidia, and basidiospores and the behavior of the nuclei at these times. It was Neuhoff (1924) who termed the structure in which nuclear fusion and meiosis occurred, the probasidium and the extensions of the basidium through which the haploid nuclei migrate into the developing spores, the epibasidia. The term commonly employed to describe the young basidium prior to septation, is probasidium; after cruciate septa are formed, the resultant cells are termed metabasidia. Donk (1964), in an attempt to clarify these terms, has related them to the nuclear condition; the former being the stage in which nuclear fusion occurs and the latter the stage in which the fusion nucleus divides. The epibasidium, termed

Diagram I. Life cycle of *T. bambusina*.


REPETITION
A
B
C
BUDDING
EPIBASIDIUM
P
CONJUGATION TUBE
D
E
METABASIDIA
O
G_2
F_1
YEAST-LIKE PHASE
HAPLOID PHASE
G_3
DIKARYOTIC PHASE
PROBASIDIA
M
L
F_2
G_1
K
G_1
HYPHAL FUSION
H
DIKARYOTIZATION
I
J
10 μ

a sterigmal apparatus by Donk, is the out-growth on which basidiospores are borne. Whelden made a critical study of nuclei in these structures for three species of *Tremella* and, in addition, described formation of two types of conidia and various abortive structures on the tips of dikaryotic hyphae.

Whelden (1934), Kobayasi (1939), and Bandoni (1961) observed the yeast-like stage and formation of conjugation tubes in several species of *Tremella*, illustrating these much as Brefeld and Möller did more than 50 years before. The study of the formation of the dikaryon by conjugation followed by conjugation studies on any member of the genus, was not made until Bandoni (1963) performed experiments on *T. mesenterica* and reported an unusual tetrapolar type mating system. A tetrapolar system reported by Kobayasi and Tubaki (1965) for *T. fuciformis* Berk. may be interpreted as résembling that of *T. mesenterica*.

An interesting aspect of the compatibility studies, was the experimental evidence showing the initiation of the sexual process by diffusible substances termed conjugation hormones (Bandoni, 1965). He demonstrated that the induction of conjugation tubes in *T. mesenterica* was primarily determined by heterozygous alleles "A" and "a" at a single incompatibility locus and that tube formation is controlled by two class specific inducing substances. Class "A" mating type initiated conjugation tubes in response to the continuous secretion of conjugation hormone(s) by the opposite mating type "a",

and the tube formation in "a" was dependent upon the secretion of substance(s) by the "A" mating type. Conjugation tube formation and fusion appear to occur whenever "A" and "a" cells are paired. The subsequent development of the dikaryotic phase appeared to be controlled by other factors which Bandoni labeled "B" factors.

Flegel (1968) investigated the conditions of conjugation for *T. encephala* Pers. and *T. subanomala* Coker, and the previously studied *T. mesenterica*. He reported that the optimal pH for the growth of the haploid phase is identical to that for growth of conjugation tubes. The substance(s) initiating conjugation tubes in the two additional species behaved similarly to that of *T. mesenterica* studied earlier by Bandoni. Flegel confirmed that these substances are required continuously for the growth of conjugation tubes and, furthermore, that tube growth is terminated with the removal of the hormone.

While studies on conjugation and formation of the dikaryon in *Tremella* have been published, studies on the subsequent formation of basidia, basidiospores and the development of the basidiocarp have not. Among members of the entire family Tremellaceae, I could find reports of basidiocarps in culture for only four species: *Exidia recisa* (Ditm.) Fr., *E. nucleata* (Schw.) Burt, (Barnett, 1937); *Aporpium caryae* (Schw.) Teixeira and Rogers, (Macrae, 1955); and *Tremella fuciformis* Berk., (Kobayasi and Tubaki, 1965).

Apparently in Formosa, *T. fuciformis* is easily cultured and is grown commercially on sawdust or sawdust mixtures (T. W. Flegel and Ping-Chao Chen, personal communication with Dr. R. J. Bandoni). Examination of several basidiocarps so produced showed no fertile basidia or basidiospores.

III. The species *Tremella bambusina* Sacc.

Using available keys, the fungus collected for use in this study, easily keys to *Tremella tubercularia* (Berk.) Berk. The description of *T. tubercularia* by Martin (1952), closely fits the collections reported in this paper. This name, however, is no longer considered valid (Massee, 1892; Massee and Crossland, 1905; Bandoni, pers. comm.) and a search of the literature for published species that could represent the local forms was made.

There are few reports of tuberculate species of *Tremella* associated with Pyrenomycetes; however, the growth of *Tremella* with other fungi is common and the inconspicuous association with submersed or partially erumpent perithecia could easily go unnoticed.

Of over 25 tuberculate species listed by Saccarado (1888, 1925) three appear to be similar to the species studied here. Of these, *T. neglecta* Tulasne, cannot be validly compared because of the brief description, lack of spore observations by the Tulasnes (1872, 1873), and because of the lack of authentic material. *T. pyrenophila* Trav. & Migl. (and the type of the synonymous *Sebacina globospora* Whelden) appears a dis-

tinct and separate species (Bandoni, pers. comm.). Bandoni's notes (pers. comm.) on the type of *T. bambusina* Sacc. listed by Saccarado (1925), correspond closely to the description of this species. I feel at this time, on the basis of these notes and the reports listed above, that the name *Tremella bambusina* Sacc. should be tentatively assigned to the species studied here. A reevaluation of the validity of this name for the local species can be made only after study of authentic *T. bambusina* cultures.

For future reference, the collections from British Columbia studied in this thesis and identified as *Tremella bambusina,* are described as follows:

The basidiocarps (Figs. 1, 2, 3) are at first tuberculate, up to (1-) 2-4 (-7) mm. broad, expanding and often becoming irregularly pulvinate and later spreading across the surface. Basidiocarps become cerebriform or more often simply convoluted, with the maximum height being partially dependent upon the moisture content, being 1-2 (-5) mm. Individual basidiocarps often anastomose with others to become up to (2-) 4-5 (-20) mm. broad. The color varies from pale sordid transluscent white, pinkish or cream-color to Light Cadmium, Cadmium-Yellow or Mustard Yellow but dries darker (colors from Ridgway, 1912). The basidiocarps appear gelatinous when young and fresh but often become watery-gelatinous in senescence or when soaked for long periods. The basidiocarps dry quickly forming a vernicose film on the substrate surface. With drying, there is an apparent sus-

Figures 1 - 3

Basidiocarps of *T. bambusina* on natural substrate.

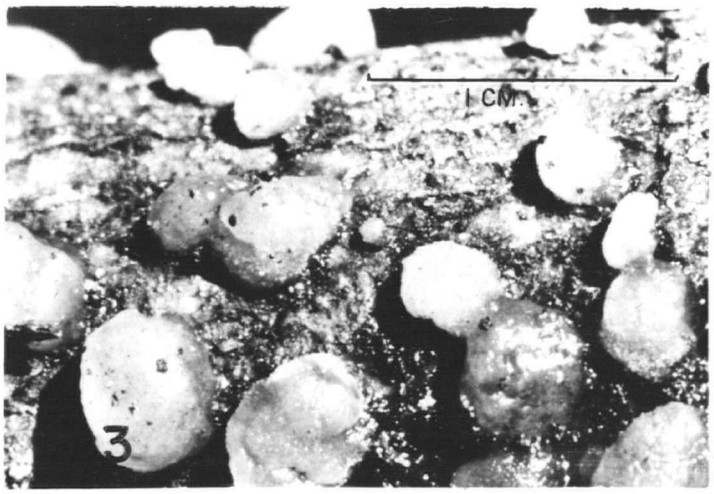
Fig. 1. Collection SGB 620, growing from *Valsa* sp. perithecia on leaves of *Thuja plicata*.

Fig. 2. Collection SGB 619, growing from *Valsa* sp. perithecia on *Rubus spectabilis*.

Fig. 3. Collection SGB 645, growing from *Diaporthe* sp. perithecia on *Cornus nuttalli*. Variation in shade indicates color variation from cream to yellow.


1 CM.
1


METRIC 1
2
2


1 CM.
3

pension of growth and spore production; the basidiocarp may revive again under wet conditions, rapidly imbibing water and returning to normal growth and spore discharge. The longevity of the basidiocarp in nature is unknown, but from observations in culture, it could be several months. All collections studied have been found to arise from the perithecial cavity of accompanying species of Valsaceae.

The hymenium is amphigenous on the exposed portion. Some small intercalary basidium-like structures, arising from clamp connections, are commonly found scattered below the hymenial area. The hyphae are compactly arranged, more or less radiating out from the subtending perithecium. Clamp connections are abundant and often give rise to hyphal branches; double clamp connections are occasionally seen (Fig. 4). Basidia formed at the terminal end of the hyphal strand are at first clavate, then subspherical to sphaero-pedunculate (not myxarioid, see Donk, 1966). A second basidium is usually present from the clamp at the base of the terminal basidium (Fig. 4, 5). Probasidia at early stages of development are easily distinguished from the hyphae because of their size, shape and the increased stainability of the protoplasm. The mature probasidia are (10-) 12-16 (-20) μ. in diameter X 20-40 (-50) μ. long; they are cruciate septate with the septa oblique (Fig. 6). The epibasidia extend up between other basidia and hyphae, to the hymenial surface. The length of the epibasidium varies depending upon the apparent stage of development and the distance from the pro-

Figures 4 - 9

Microscopic features of developing basidia and basidiospores, from basidiocarps found in nature.

Fig. 4. The characteristic form and location of the second basidium arising from the clamp connection at the base of the terminal basidium. Note what appears to be double and single clamp connections.

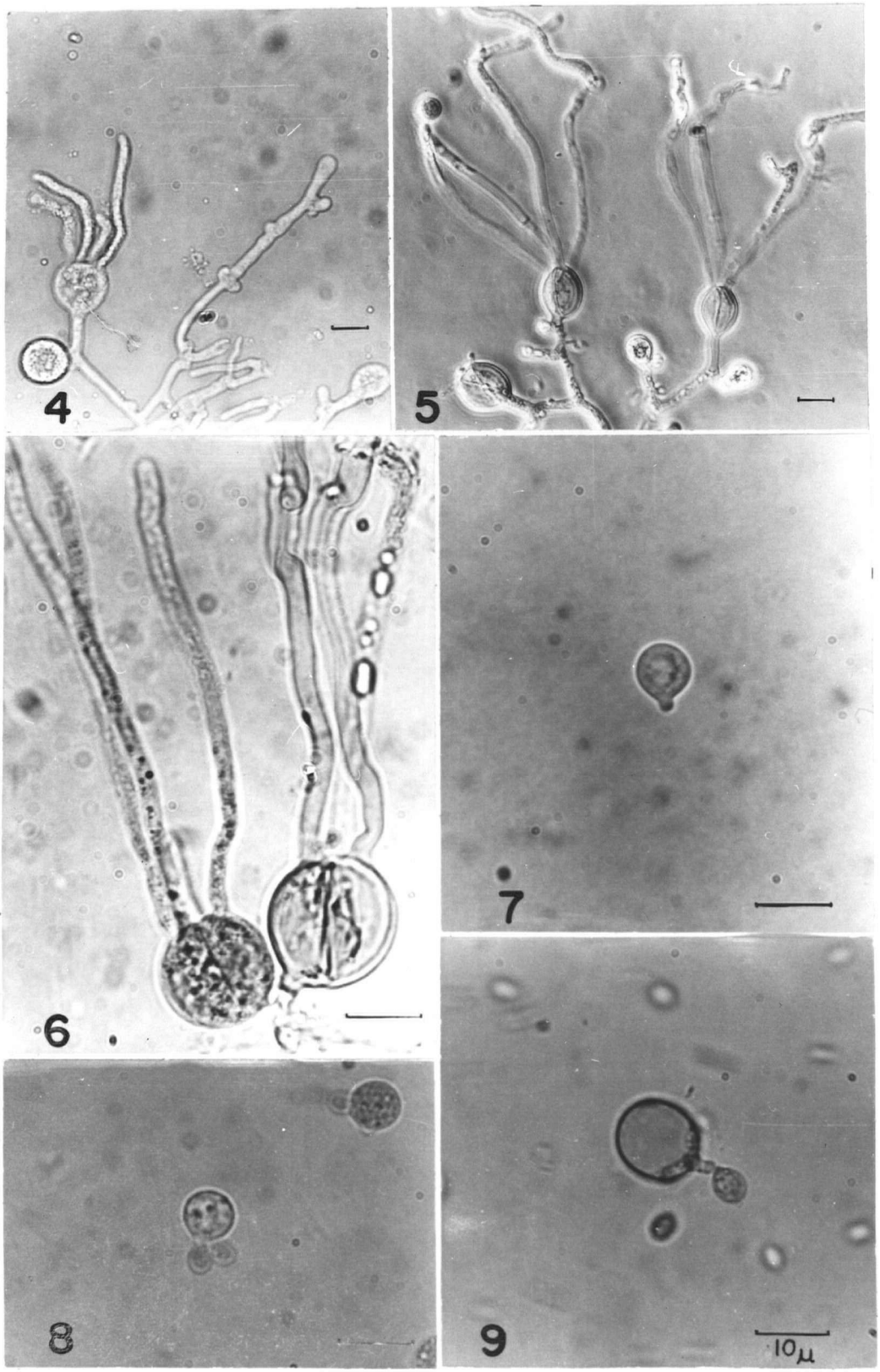
Fig. 5. Probasidia showing the contorted epibasidia at different stages of development, one with a developing basidiospore.

Fig. 6. Basidia clearly showing the characteristic oblique septa. One section of the probasidium and the associated epibasidium are almost empty of protoplasm.

Fig. 7. Basidiospore with broad, slightly eccentric, apiculus.

Fig. 8. Basidiospore germinating by budding.

Fig. 9. Basidiospore germinating by repetition.


4
5
6
7
8
9
10μ

basidium to the surface of the basidiocarp. They are cylindrical, (1.5-) 2 (-3.5) μ. in diameter and in some instances reach a length of over 100 μ. The production of epibasidia by the four cells of a basidium are not necessarily synchronous (Fig. 6). The basidiospores are one-celled, smooth, hyaline and sub-globose, (6-) 7-9 (-10) μ. in diameter with a broad rounded apiculus, the latter 2-3 μ. wide and 1-2 μ. long (Fig. 7). Basidiospores are infrequently found on the surface of the basidiocarp, where they germinate by budding, or by repetition (Fig. 8, 9). No regular formation of hymenial conidia was observed on basidiocarps collected from nature.

MATERIALS AND GENERAL METHODS

I. Source of materials

Erect dead canes of *Rubus spectabilis* Pursh, salmonberry, provided the most reliable and constant source for study material. Collections were easily made during or immediately after a heavy rain. If collections were not made at these times, the basidiocarp became almost imperceptible in the field because of rapid drying. Other sources of material were the stems of *Sambucus pubens* Michx., elderberry; *Cornus nuttallii* Aud. ex T. & G., pacific dogwood; *Acer circinatum* Pursh, vine maple; *Alnus rubra* Bong., red alder; and from undetermined species of *Salix*. It has also been collected from the stems of the conifers *Pseudotsuga menziesii* (Mirbel) Franco, Douglas fir; *Tsuga heterophylla* (Raf.) Sarg., western red cedar. (See Appendix A: Collections Studied). Despite this range of substrate species, *T. bambusina* was observed in all collections, to grow directly from the perithecia of species of either *Valsa* or *Diaporthe*.

All isolates of *T. bambusina* used in this study were made from material collected in the vicinity of Vancouver, British Columbia, Canada. Data on each collection and a list of haploid cultures from single spore isolates are recorded in Appendix A: Isolates. All cultures were maintained in 150 X 60 mm. culture tubes, on malt-yeast soytone agar medium

(MYS) (for formula, see Appendix B: Media), capped, then sealed with Parafilm. Viable cultures were maintained without transfer, for over a year at room temperature and over 2 years at 5° C., with no additional care. Reference cultures of all isolates were maintained under mineral oil at room temperature; representative mating types listed in Table VIII are deposited in the Mycological Culture Collection of the University of British Columbia. Dried collections from which isolates were obtained are deposited in the Mycological Herbarium of the University of British Columbia.

II. Study of dried herbarium collections

The dried collections from the herbarium of Dr. R. J. Bandoni as well as those collected by myself, were examined and are listed in Appendix A: Collections Studied. The basidiocarps were quickly revived by placing a few drops of distilled water on them. Thin free-hand sections were made using a razor blade and these sections were placed upon a microscope slide in a 2% solution of KOH (See Appendix C: Stains and Solutions) to soften the material and facilitate its crushing and staining. A drop of Congo Red (See Appendix C: Stains and Solutions) was added to the material and the excess removed by blotting with a paper towel. A microscope coverslip placed over the section and gently pressed, spread the material out for observation. Phloxine (See Appendix C: Stains and Solutions) was added for additional staining and as a temporary mounting medium. Microscopic observations and

measurements were then made. Slides of associated pyrenomycetes were prepared as crushed mounts in KOH or from freehand sections. Drawings were prepared using a Leitz microscope and drawing attachment; photographs were taken with a Leitz-orthomat camera. The line inscribed in each photomicrograph represents a distance of 10 μ. Photographs from cultures of germinating basidiospores (Figs. 7-9, 14-19), conjugation tubes (Figs. 11-12, 29-35), hyphae (Figs. 36-41) and the basidiocarps (Figs. 43-45) were taken *in situ*.

III. Obtaining single spore isolates

A portion of the substratum bearing a basidiocarp of *T. bambusina* was cut off and taped to the lid of a sterilized Petri plate. The sporulating basidiocarp was attached by masking tape over a pad of several thicknesses of moistened filter paper and arranged so that only the hymenial surface was exposed. The lid was then inverted over MYS medium. The basidiocarp was so placed, that by turning the lid to new positions, additional sterile surfaces were provided for the deposition of spores. Position changes were made, depending upon how rapidly the spores were discharged, to insure sufficient spore separation. When spores had been deposited, the lid with the suspended basidiocarp was replaced by a sterile lid and the plate incubated at 15° C. for 2 days, then examined. If a period of incubation was not allowed, detection of single spore colonies was difficult. Colonies of several cells, each representing a haploid clone resulting

from the germination of a single basidiospore, were observed under 14-60 magnification of a zoom type B.&L. stereo microscope fitted with a 2X supplemental lens. Colonies were transferred to separate plates of MYS using a sterilized needle. A small portion of the subtending agar had to be included with each to ensure the transfer of isolates. Later it was observed that if the culture was allowed to dehydrate slightly, the clones could be more easily picked up with a needle. Isolates were grown at 15° C. for approximately 1 week, transferred to slants of MYS and retained as stock cultures.

Raper (1966) suggested the term 'strain' be restricted to the homokaryotic isolates, which in *Tremella*, are represented by the yeast-like phase. The term 'stock' was suggested for use in describing dikaryotic isolates. These terms are used similarly in the present study.

IV. Compatibility studies

Studies to determine the mating system and subsequent crosses were carried out on Conjugation medium (CJA) (See Appendix B: Media). Preliminary experiments resulted in the standardization of inoculation techniques described here. Inoculum was prepared 1 week in advance and small amounts of each strain were spotted, with a sterilized inoculating loop, near but not touching each other on the solidified medium surface. These two strains were then thoroughly mixed using a sterilized inoculating loop. Usually four tests were made

on one plate, all equidistant between side and center of the plate and within each quarter of the medium surface. No attempt was made to control the exact depth of the medium. Following inoculation, cultures were incubated at 15° C. with alternating 12-hour dark/light periods (Percival Incubator). Light was supplied by a 20 watt, "cool white", 25 inch fluorescent lamp (G.E. or Westinghouse).

All isolated strains from each collection of *T. bambusina* were paired in all possible combinations. All pairings were made at least twice. At the end of 1 month's incubation, the yeast-like colony was scraped from the agar surface with a coverslip. Microscopic examination was made directly upon the agar by placing the uncovered Petri plate upon the microscope stage. Generally the placement of a coverslip over the observed area was not necessary for the determination of compatibility or the presence of the dikaryotic phase.

V. Culture techniques and growth conditions

A. Haploid phase

Observations on the germination of basidiospores and the nature of the haploid phase were carried out on MYS, on the surface of basidiocarps, or moist coverslips or inner lid surfaces of Petri plates where discharged basidiospores were deposited. *In situ* photographs, drawings and measurements, under oil immersion, were made with a coverslip placed over the desired area of the agar or glass surface.

All experiments in which chemically defined media were

used (SM series) (See Appendix B: Media), including separate studies of nitrogen, carbon, and vitamin nutrition, the effects of calcium on growth, growth curves and correlation measurements, were carried out in chemically cleaned Pyrex glassware. All flasks and mixing vessels were cleaned with a potassium dichromate-sulfuric acid cleaning solution (See Appendix C: Stains and Solutions), and rinsed thoroughly three times in glass distilled water before use. Water was obtained from a Corning AG-la glass still and was stored in a plastic carboy. Pyrex Erlenmeyer flasks (125 ml.) were used as culture vessels. Each flask contained 25 ml. of liquid medium, unless otherwise specified, and was capped with a double sheet of aluminum foil. Cultures were inoculated from a homogeneous suspension of the haploid cells that had been taken from colonies grown 7 days on MYS at 15° C. Care was taken to insure an even distribution of cells and measured volumes or drops of this suspension were pipetted directly into the culture flasks as inoculum. A 1 ml. measuring pipette was used and identical measures of inoculum of any one strain were added to each series. Medium for specific tests was prepared in one large flask, mixed thoroughly and divided among individual flasks prior to adding the experimental materials or concentrations. Cultures were grown 3 to 7 days at room temperature on a recriprocating shaker at 80 cycles per minute.

To determine growth, the cultures were measured for

optical density using a B. & L. Spectronic 20 spectrophotometer (blue sensitive phototube, wavelength of 550 mμ). Growth curves were obtained by measuring growth rates in nepheloculture flasks. These were prepared by attaching cuvette tubes with plastic tubing to 250 ml. side arm filtering flasks. pH was determined with the aid of a Radiometer pH meter, model 28.

Various modifications of Lilly and Barnett's (1951) basic synthetic medium (SMA) (See Appendix B: Media), led to the formulation of synthetic medium B(SMB), C (SMC), and D (SMD) (See Appendix B: Media).

Calcium effects: The numerous and varied calcium experiments necessitates placing a description of the specific methods and materials used, with results in the section "Specific Techniques, Results and Observations".

Nitrogen requirements: Experiments on the nitrogen requirements of *T. bambusina* were based on SMD. No attempt was made in these experiments to maintain the pH within narrow limits by buffer systems. There was no detectable difference in initial optical densities of these media, even though they differed somewhat in ingredients. The nitrogen compounds were prepared so that they would yield the amount of nitrogen equivalent to that in two grams of asparagine. An increase of glucose to 15 g./liter provided an excess of carbon during the 4-day growth period so that the carbon, possibly available from ammonium tartrate, $(NH_4)_2C_4H_4O_6$, would be minimized.

All other elements of the medium were provided as indicated in individual formulae.

Carbon requirements: Equivalent amounts of carbon were calculated for four common carbon sources and included as a modification of SMD in an attempt to determine which were usable. Measured optical densities of the media were identical prior to autoclaving. Microscopic observations and counts were employed as a check for growth. No buffers were added to the media.

Vitamin requirements: Selected vitamins listed in Table XV were made up in a 30% ethyl alcohol solution, 12 hours prior to use. Vitamin free SMC was autoclaved and cooled, then vitamins were added separately, each at a level suggested by Whisler (1962), of 200 μg./liter, except for biotin, which was added at 100 μg./liter. All other media requiring the addition of vitamins were prepared in a manner similar to that recorded in Appendix B: Media. These vitamins were added at 100 μg./liter except biotin which was added at 5 μg./liter. In addition a comparison of the growth in medium made up with vitamins added before and with vitamins added after autoclaving, was carried out. Strains growing 1 week on SMC to which no vitamins had been added, were inoculated in each flask in equal measures. Flasks were incubated at room temperature in shake culture for 3 or 4 days.

PH tolerance: To determine the pH tolerance, liquid conjugation medium (CJL) (See Appendix B: Media) was prepared

at pH values of 4.0 to 8.5 at 0.5 intervals, by the addition of 1N NaOH or 1N HCl. Inoculum of strains was prepared by growing each for 1 week on MYS prior to inoculation. To reduce rapid initial changes in pH, 100 ml. of medium was used in each flask instead of the previously used volume of 25 ml. After incubating 3 days on a shaker at room temperature, the pH was again recorded and optical densities measured. No buffers were added.

B. Conjugation phase

Tests were made with pairs 523 X 2, 711 X 5, and 12 X 5, to determine the effects of various physical conditions on conjugation tube formation. Five replicates of each cross were carried out on CJA for each of nine physical conditions. All inoculum used had been streaked on CJA 2 days prior to inoculation and all cultures were incubated at 18°C. prior to testing of each condition. After the optimum age, amount, and degree of mixing of inoculum had been ascertained, these were standardized and used in determining medium conditions, light and temperature levels for best conjugation tube growth. To test the effect of medium age, medium was prepared and poured into Petri plates that were either used the next day or stored in metal containers for 1 month at 18° C., prior to inoculation. New medium was prepared on the day prior to inoculation of the stored plates and was used as an additional comparison.

The comparison of effects of inoculum age on conjugation

was made by streaking selected 28 day-old strains on MYS 2 days prior to use; these were then reinoculated and mixed in compatible combinations on one-half of each plate and were compared with the original 28 day-old strains mixed in the same combinations on the opposite half. Observations were then made at the end of 2 and 4 days incubation.

All photographs, measurements, descriptions and observed characteristics of the development of conjugation tubes were made with the same compatible pairs used to test physical requirements as well as 523 X 5, 15 X 3, and 523 X 522. These pairs were chosen from the most consistently and abundantly producing tubes in the compatibility tests. Each half of each plate was spotted with a compatible pair of strains. Consistency in location, the amount of area covered, the degree of mixing, inoculum amount, age of culture and medium age was closely maintained. Plates were incubated at 15° C. under alternate 12-hour periods of light and dark, and were placed approximately 15 cm. below the florescent light source.

Hormone studies: In determining the hormone characteristics, four compatible pairs were used: 523 X 522, 523 X 2, 15 X 3 and 711 X 5. Five replicates of each test were made and patterned after the technique described by Bandoni (1963). Cellulose dialysis membrane with a wall thickness of 0.0016 mm. (General Biological Supply House) was cut into 5 cm. squares, autoclaved in distilled water and transferred aseptically to CJA plates. Observations were made daily up to 7

days after compatible pairs were brought together. To determine diffusibility of conjugation hormone(s) through dialysis membrane, each strain was inoculated on conjugation medium and allowed to grow for 5 days. This was then covered by dialysis membrane and a second compatible strain was placed above the original but separated by the membrane. The reciprocal of each pairing was also carried out. To determine if the conjugation hormones were produced continuously, each of eight strains of *T. bambusina* was inoculated on separate dialysis membranes placed over agar medium on the 1st, 3rd, and 5th day. One additional day was allowed for incubation after the 5th day; the membrane was then removed along with the strain and the same medium area was reinoculated with a compatible strain.

CJA was used as the medium in determining the maximum diffusion distance of the conjugation hormone, as well as the reaction at the tips when hyphae approached one another. Compatible strains were streaked across the medium at an angle of approximately 35° with each other, forming a "V". The two streaks terminated at a point near one edge of the medium and were separated approximatly 40 mm. on the opposite edge. Care was taken to ensure that these opposite types were not mixed except at the termination of the streaks where they met. Locations were noted where tube initiation occurred and the minimum distance from the tube to the nearest portion of the streak of the opposite type was measured.

Heat lability tests of the hormones were carried out by growing strains on CJA or MYS for 4 or 7 days at 15° and 20° C. These cultures were then autoclaved at 15 lbs./15 min., allowed to cool quickly in a 10° C. refrigerator, and then reinoculated with a compatible strain at various places across the medium surface, including next to the initial colonies. Three methods of applying the initial inoculum were employed: 1), drops of one strain dispersed in distilled water were placed on the agar and spread uniformly over the surface with the aid of a modified slide-ringing turntable and a sterilized "L" shaped glass rod; 2), application of inoculum was applied to approximately one square centimeter on the center of the medium surface; 3), several spots of inoculum were placed on the surface but separated from one another by a distance of at least 2 cm.

C. Dikaryotic mycelial phase

Microscopic studies of the morphology and development of the dikaryotic phase and the formation of basidia and basidiospores were carried out *in situ* as described for the haploid phase. Alternatively fixed-stained slides were prepared of the mycelial phase. Material was stained, crushed and mounted in a manner similar to that described for the study of dried specimens. From conjugation studies, crosses 12 X 15, 2 X 15, 523 X 2, 543 X 2 and 711 X 5 were among those appearing to produce basidia most consistently and abundantly. These stocks were chosen for the studies of basidium, basidio-

spore, and basidiocarp formation. For any one series of experiments, inoculum consisted of 2 mm. square blocks containing the dikaryotic hyphae. These blocks were cut from the leading edge of a colony or basidiocarp growing on MYS at 15° C. under alternate 12-hour light/dark periods. Cultures were then incubated under the same conditions of temperature and light unless otherwise specified. The experiments on the effects of temperature on the growth of the dikaryotic phase were made in a temperature gradient box placed in a 3° C. incubator. Heat was supplied by a 7.5 watt lamp, loosely covered with foil, providing a series of temperature levels between 11° and 17° C. (± 2° C.). The temperature fluctuation was such that only a rough estimate of optimum temperatures could be obtained.

In other experiments, extracts of *Valsa* species were prepared by two methods. Both involved the physical dissection of groups of perithecia from portions of stem material that also bore basidiocarps of *T. bambusina*. In one instance the *Valsa* perithecia along with small portions of the intact stem, were autoclaved in 10 ml. of MYS medium. The other method involved placing perithecia of *Valsa* in 95% ethyl alcohol solution for 2 weeks, then placing the sterilized perithecia and spores directly on inoculated plates of the dikaryotic phase of *T. bambusina*.

Valsa cultures were obtained from freshly collected canes of Salmonberry that also bore basidiocarps of *T. bambu-*

sina. The spores extruded from the perithecia were picked up with an inoculating needle and placed directly upon MYS; these were retained in stock culture. Spore producing pycnidia were formed in culture but perithecia were not.

SPECIFIC TECHNIQUES, RESULTS AND OBSERVATIONS

I. Compatibility studies

Strains of *T. bambusina* originating from single spore isolates were retained as stock cultures. Isolated strains of each collection reported in Table XXII (See Appendix A: Collections Studied), were paired in all possible combinations. After 30 days incubation, paired cultures could be classed into three groups: 1) cultures showing only a continuation of growth by budding (0), 2) cultures containing budding cells and conjugation tubes (T), and 3) cultures containing the budding cells, conjugation tubes and dikaryotic mycelium (TCl).

The haploid budding cells observed (1) (Fig. 10) could not be distinguished from the original haploid strains. The conjugation tubes (2) (Fig. 11) lacked clamps, were generally short and were found near the surface of the medium, penetrating the agar only slightly. They were mostly unbranched and sparingly septate, and did not grow extensively. The presence of clamp connections was taken as an indicator of the dikaryotic phase. The dikaryotic hyphae (3) (Fig. 12) were septate, branched, and penetrated into the agar medium to a considerable degree. The dikaryotic hyphae consistently were more extensive around the colony edge than at its center (Fig. 13).

A. Intra-strain crosses

The results of the pairings of each isolate of seven basidiocarps representing five collections, were analysed

Figures 10 - 13

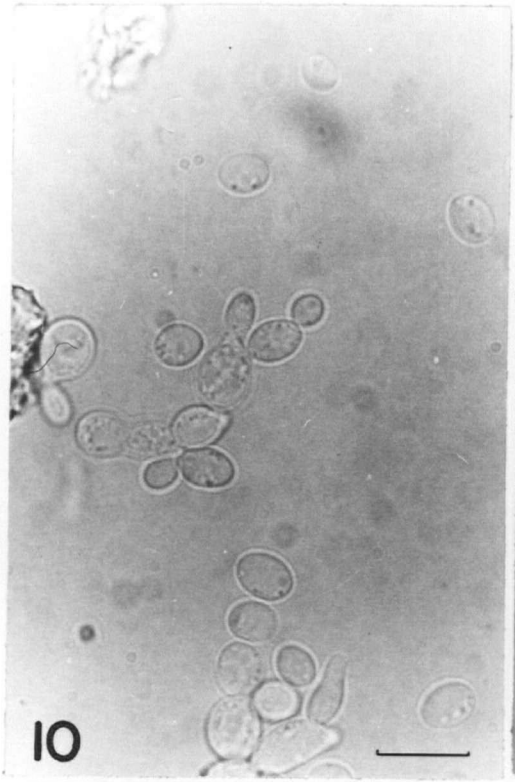
Haploid phase and dikaryotic phase.

Fig. 10. Haploid phase, showing yeast-like cells. (0)

Fig. 11. Haploid phase, showing conjugation tubes and budding cells. (T)

Fig. 12. Dikaryotic phase, showing hyphae with clamps. (TC1)

Fig. 13. Agar plate (CJA) after removal of two surface colonies. The upper half bore a colony of two incompatible budding strains. The lower half contained paired compatible strains. The embedded dikaryotic mycelium is visible (arrow).



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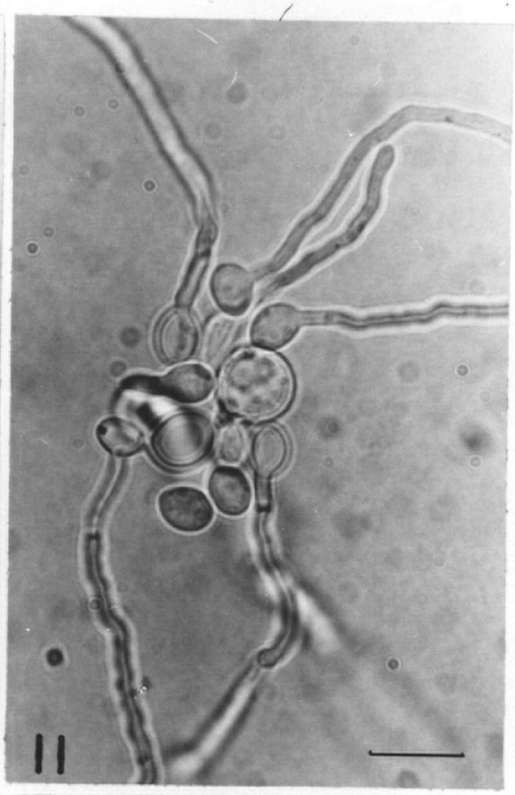

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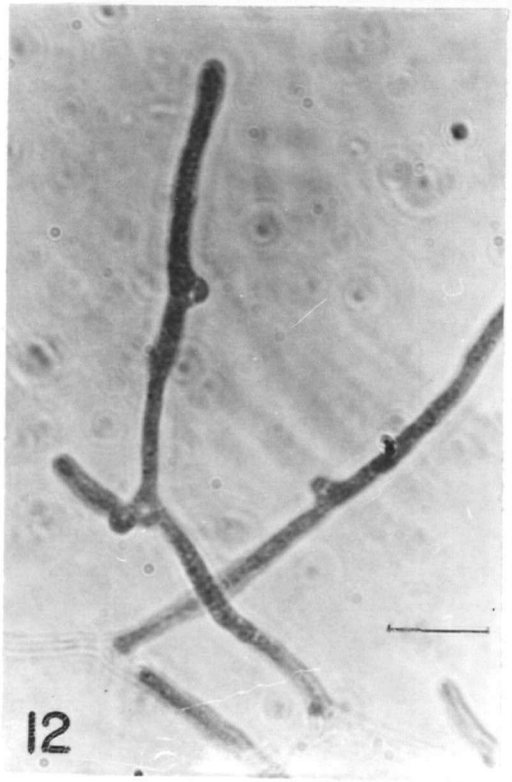

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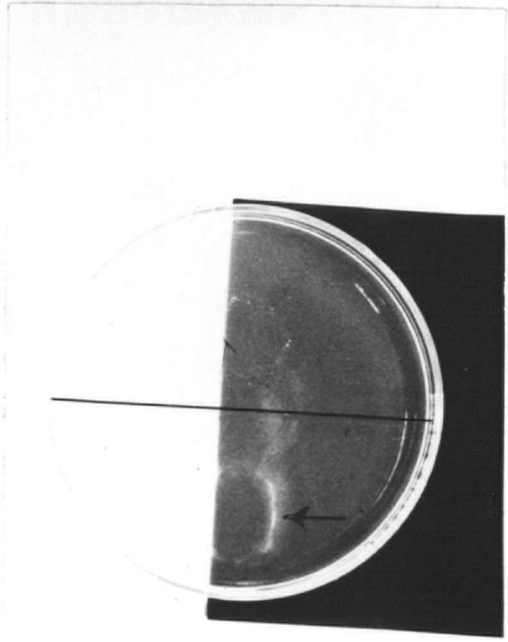

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(Tables I, II, III, IV, V, VI, VII). A tetrapolar compatibility pattern similar to that reported by Bandoni (1963) emerged. The four mating types were assigned a specific "A" or "B" incompatibility factor of a tetrapolar scheme. In no instance was fusion detected between pairs not compatible for both the "A" and "B" factors.

The terms "A" and "a" represent the locus regulating formation of conjugation tubes; "B_1", "B_2", etc., represent the locus controlling establishment of dikaryon by fusion of compatible tubes.

B. Inter-strain crosses

From the seven collections of *T. bambusina* listed in Table XXIII, strains representing the four compatibility types from each collection were chosen and paired in all possible combinations. Pairings between strains from a single basidiocarp resulted in a pattern identical to that already established. Those pairings between strains from different basidiocarps all resulted in the formation of a dikaryotic phase where paired isolates were previously calculated to contain unlike "A"s. Thirteen different "B" factors were obtained, and all were compatible. All strains with the suggested "A" and "B" genotypes are listed in Appendix A: Table XXIII.

II. Haploid phase

A. Germination of basidiospores

One-celled basidiospores readily germinated within a few

Table I. Results of pairing all isolates from collection SGB 620A.

		AB_1	AB_2			aB_1		aB_2
		1	3	4	5	6	7	2
AB_1	1	0	0	0	0	T	T	TCl
AB_2	3		0	0	0	TCl	TCl	T
	4			0	0	TCl	TCl	T
	5				0	TCl	TCl	T
aB_1	6					0	0	0
	7						0	0
aB_2	2							0

KEY
0=Budding cells only
T=Conjugation tubes
TCl=Tubes with clamps (Dikaryotic phase)

Table II. Results of pairing all isolates from collection SGB 620B.

		AB_3	AB_4	aB_3		aB_4	
		12	8	9	11	10	18
AB_3	12	0	0	T	T	TCl	TCl
AB_4	8		0	TCl	TCl	T	T
aB_3	9			0	0	0	0
	11				0	0	0
aB_4	10					0	0
	18						0

Table III. Results of pairing all isolates from collection SGB 620C.

		AB_5	AB_6	aB_5		aB_6	
		14	*	13	15	16	17
AB_5	14	0	-	T	T	TCl	TCl
AB_6	*		-	-	-	-	-
aB_5	13			0	0	0	0
	15				0	0	0
aB_6	16					0	0
	17						0

Table IV. Results of pairing all isolates from collection SGB 625A.

		AB_7				AB_8					aB_7		aB_8					
		523	524	534	535	527	530	533	536	537	531	538	521	522	525	528	529	532
AB_7	523	0	0	0	0	0	0	0	0	0	T	T	TCl	TCl	TCl	TCl	TCl	TCl
	524		0	0	0	0	0	0	0	0	T	T	TCl	TCl	TCl	TCl	TCl	TCl
	534			0	0	0	0	0	0	0	T	T	TCl	TCl	TCl	TCl	TCl	TCl
	535				0	0	0	0	0	0	T	T	TCl	TCl	TCl	TCl	TCl	TCl
AB_8	527					0	0	0	0	0	TCl	TCl	T	T	T	T	T	T
	530						0	0	0	0	TCl	TCl	T	T	T	T	T	T
	533							0	0	0	TCl	TCl	T	T	T	T	T	T
	536								0	0	TCl	TCl	T	T	T	T	T	T
	537									0	TCl	TCl	T	T	T	T	T	T
aB_7	531										0	0	0	0	0	0	0	0
	538											0	0	0	0	0	0	0
aB_8	521												0	0	0	0	0	0
	522													0	0	0	0	0
	525														0	0	0	0
	528															0	0	0
	529																0	0
	532																	0

Table V. Results of pairing all isolates from collection SGB 625B.

		AB_9			AB_{10}	aB_9		aB_{10}	
		524	544	545	543	541	547	546	548
AB_9	542	0	0	0	0	T	T	TCl	TCl
	544		0	0	0	T	T	TCl	TCl
	545			0	0	T	T	TCl	TCl
AB_{10}	543				0	TCl	TCl	T	T
aB_9	541					0	0	0	0
	547						0	0	0
aB_{10}	546							0	0
	548								0

Table VI. Results of pairing all isolates from collection SGB 645.

		AB_{11}			AB_{12}			aB_{11}					aB_{12}
		618	619	622	613	614	615	612	616	617	620	621	611
AB_{11}	618	0	0	0	0	0	0	T	T	T	T	T	TCl
	619		0	0	0	0	0	T	T	T	T	T	TCl
	622			0	0	0	0	T	T	T	T	T	TCl
AB_{12}	613				0	0	0	TCl	TCl	TCl	TCl	TCl	T
	614					0	0	TCl	TCl	TCl	TCl	TCl	T
	615						0	TCl	TCl	TCl	TCl	TCl	T
aB_{11}	612							0	0	0	0	0	0
	616								0	0	0	0	0
	617									0	0	0	0
	620										0	0	0
	621											0	0
aB_{12}	611												0

Table VII. Results of pairing all isolates from collection SGB 655.

		AB_{13}		AB_{14}							aB_{13}			aB_{14}				
		705	710	702	703	709	712	713	714	715	701	706	717	704	707	708	711	716
AB_{13}	705	0	0	0	0	0	0	0	0	0	T	T	T	TC1	TC1	TC1	TC1	TC1
	710		0	0	0	0	0	0	0	0	T	T	T	TC1	TC1	TC1	TC1	TC1
AB_{14}	702			0	0	0	0	0	0	0	TC1	TC1	TC1	T	T	T	T	T
	703				0	0	0	0	0	0	TC1	TC1	TC1	T	T	T	T	T
	709					0	0	0	0	0	TC1	TC1	TC1	T	T	T	T	T
	712						0	0	0	0	TC1	TC1	TC1	T	T	T	T	T
	713							0	0	0	TC1	TC1	TC1	T	T	T	T	T
	714								0	0	TC1	TC1	TC1	T	T	T	T	T
	715									0	TC1	TC1	TC1	T	T	T	T	T
aB_{13}	701										0	0	0	0	0	0	0	0
	706											0	0	0	0	0	0	0
	717												0	0	0	0	0	0
aB_{14}	704													0	0	0	0	0
	707														0	0	0	0
	708															0	0	0
	711																0	0
	716																	0

Table VIII. Results of pairing each of four representative mating types from seven collections.

	Thuja plicata Valsa 620 A				Thuja plicata Valsa 620 B				Thuja plicata Valsa 620 C				Rubus spectab. Valsa 625 A				Rubus spectab. Valsa 625 B				Cornus nuttallii Diaporthe 645				Pseudotsuga menz. Valsa 655			
	1	5	2	6	12	8	11	10	14	*	13	16	524	533	538	522	545	543	541	546	619	614	617	611	710	712	706	711
1	0	0	T	TC1	0	0	TC1	TC1	0		TC1	TC1	0	0	TC1	TC1	0	0	TC1	TC1	0	0	TC1	TC1	0	0	TC1	TC1
5	0	0	TC1	T	0	0	TC1	TC1	0		TC1	TC1	0	0	TC1	TC1	0	0	TC1	TC1	0	0	TC1	TC1	0	0	TC1	TC1
2	T	TC1	0	0	TC1	TC1	0	0	TC1		0	0	TC1	TC1	0	0	TC1	TC1	0	0	TC1	TC1	0	0	TC1	TC1	0	0
6	TC1	T	0	0	TC1	TC1	0	0	TC1		0	0	TC1	TC1	0	0	TC1	TC1	0	0	TC1	TC1	0	0	TC1	TC1	0	0
12					0	0	T	TC1	0		TC1	TC1	0	0	TC1	TC1	0	0	TC1	TC1	0	0	TC1	TC1	0	0	TC1	TC1
8					0	0	TC1	T	0		TC1	TC1	0	0	TC1	TC1	0	0	TC1	TC1	0	0	TC1	TC1	0	0	TC1	TC1
11					T	TC1	0	0	TC1		0	0	TC1	TC1	0	0	TC1	TC1	0	0	TC1	TC1	0	0	TC1	TC1	0	0
10					TC1	T	0	0	TC1		0	0	TC1	TC1	0	0	TC1	TC1	0	0	TC1	TC1	0	0	TC1	TC1	0	0
14									0		T	TC1	0	0	TC1	TC1	0	0	TC1	TC1	0	0	TC1	TC1	0	0	TC1	TC1
*																												
15									T		0	0	TC1	TC1	0	0	TC1	TC1	0	0	TC1	TC1	0	0	TC1	TC1	0	0
16									TC1		0	0	TC1	TC1	0	0	TC1	TC1	0	0	TC1	TC1	0	0	TC1	TC1	0	0
524													0	0	T	TC1	0	0	TC1	TC1	0	0	TC1	TC1	0	0	TC1	TC1
533													0	0	TC1	T	0	0	TC1	TC1	0	0	TC1	TC1	0	0	TC1	TC1
538													T	TC1	0	0	TC1	TC1	0	0	TC1	TC1	0	0	TC1	TC1	0	0
522													TC1	T	0	0	TC1	TC1	0	0	TC1	TC1	0	0	TC1	TC1	0	0
545																	0	0	T	TC1	0	0	TC1	TC1	0	0	TC1	TC1
543																	0	0	TC1	T	0	0	TC1	TC1	0	0	TC1	TC1
541																	T	TC1	0	0	TC1	TC1	0	0	TC1	TC1	0	0
546																	TC1	T	0	0	TC1	TC1	0	0	TC1	TC1	0	0
619																					0	0	T	TC1	0	0	TC1	TC1
614																					0	0	TC1	T	0	0	TC1	TC1
617																					T	TC1	0	0	TC1	TC1	0	0
611																					TC1	T	0	0	TC1	TC1	0	0
710																									0	0	T	TC1
712																									0	0	TC1	T
706																									T	TC1	0	0
711																									TC1	T	0	0

1 = AB_1	12 = AB_3	14 = AB_5	524 = AB_7	545 = AB_9	619 = AB_{11}	710 = AB_{13}
5 = AB_2	8 = AB_4	* = AB_6	533 = AB_8	543 = AB_{10}	614 = AB_{12}	712 = AB_{14}
2 = aB_1	11 = aB_3	15 = aB_5	538 = aB_7	541 = aB_9	617 = aB_{11}	706 = aB_{13}
6 = aB_2	10 = aB_4	16 = aB_6	522 = aB_8	546 = aB_{10}	611 = aB_{12}	711 = aB_{14}

hours after discharge. While experiments were not carried out on the factors affecting basidiospore germination, observations recorded may provide preliminary information useful for further investigations. Germination took place on agar media with pH levels of 6.2 and 7.2 (MYS, CJA) and at 15°, 20° and room temperature (approx. 21° C.). In one instance, where only one of 40 isolated basidiospores germinated, the failure was attributed to incubator temperatures reaching 30° C. over a 2 day period. While the basidiospores may have been subjected to light prior to their discharge from the basidiocarp, spore germination readily occurred in spore-drop plates placed in the dark interior of an incubator.

Three types of germination were observed: budding, repetition and conjugation tube. Budding occurred on solid media as well as in water condensed on glass surfaces (e.g. coverslips placed on medium) within the Petri plate. In such instances there appeared little or no swelling prior to germination. The buds arising directly from the basidiospore are designated as primary buds. These appear from any position on the spore wall but seldom arise from near the apiculus (Fig. 14). The first of the primary buds remain attached to the spore and give rise to numerous successive secondary buds from its distal end (Fig. 15, 16). Additional primary buds generally arise from the same area of the spore (Fig. 16) and either remain attached or break away. In all instances primary cells appear capable of continued budding. The basidio-

Figures 14 - 19

Germination of basidiospores on MYS medium. Photographed *in situ*.

Fig. 14. Five basidiospores, showing primary buds in various stages of development.

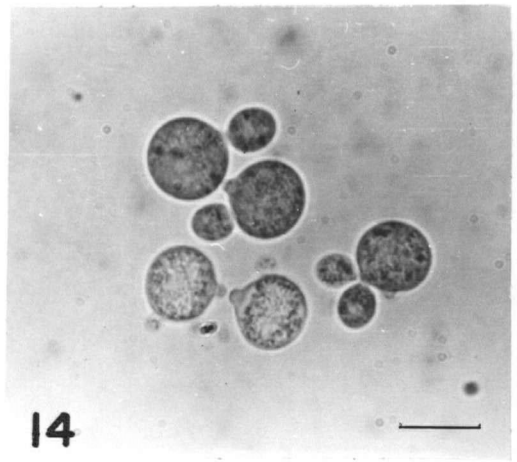
Fig. 15. A secondary bud forming at the tip of the primary cell.

Fig. 16. Two primary buds still attached to the basidiospore and forming secondary buds. Note the large eccentric vacuole.

Fig. 17 Basidiospore with prominent vacuole and numerous primary and secondary cells.

Fig. 18. Young clone of cells, including basidiospore.

Fig. 19. Basidiospore germinating directly by conjugation tube. Note adjacent colony with some cells elongated in initial stages of tube formation.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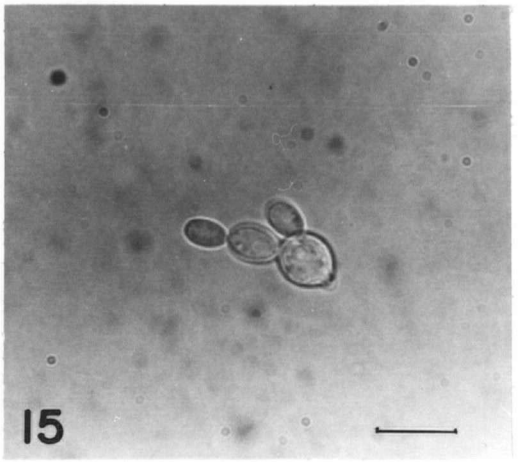
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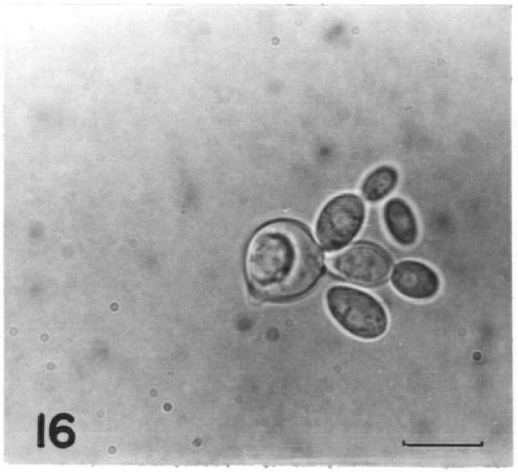
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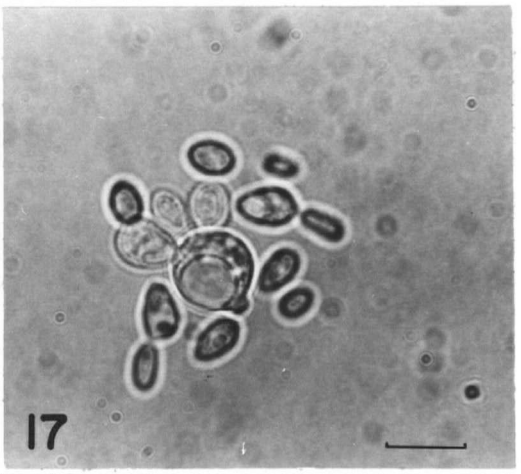
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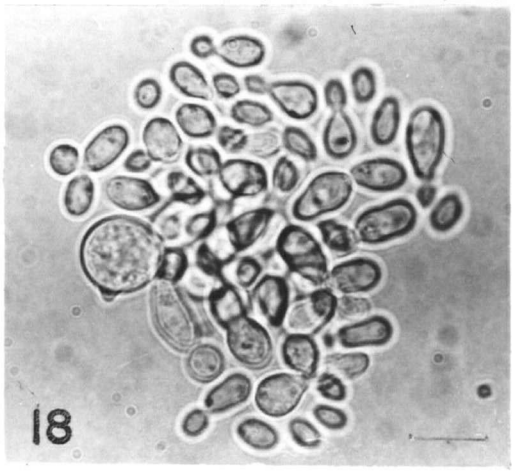
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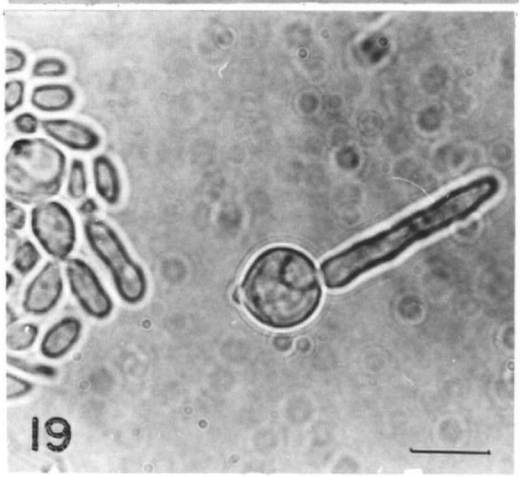
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iospore can be readily distinguished from the daughter cells by its large size and subglobose shape (Fig. 18). As more primary and secondary cells appeared, the basidiospore developed a large eccentric vacuole (Fig. 16, 17). The spore was soon obscured by the abundance of daughter cells and observations were discontinued or the colony was dispersed for further observations. When basidiospores could be found 2 weeks after initial germination, they still possessed cytoplasm and attached primary buds.

Germination by conjugation tubes (Fig. 19, 20) was observed only on agar surfaces and where compatible basidiospores were in close proximity. These conjugation tubes either fused at their tips or formed buds at their apices similar to those illustrated in Fig. 32.

Germination by repetition took place on the surface of the basidiocarp. The basidiospore developed a sterigma (Fig. 22) terminating in a secondary spore similar in appearance to the original basidiospore (Fig. 21). This secondary spore is attached at the base of a broad apiculus and is forcibly discharged. After discharge of the secondary spore, the basidiospore collapses. In some instances the sterigma appeared to revert to the formation of buds (Fig. 23, 24), while protoplasm was still present in the basidiospore (Fig. 22). In only one instance was repetition recorded apart from the hymenial surface and this was on the moist surface of a Petri plate lid of an inverted culture.

Figures 20 - 24

Germination of basidiospores from the hymenial surface.

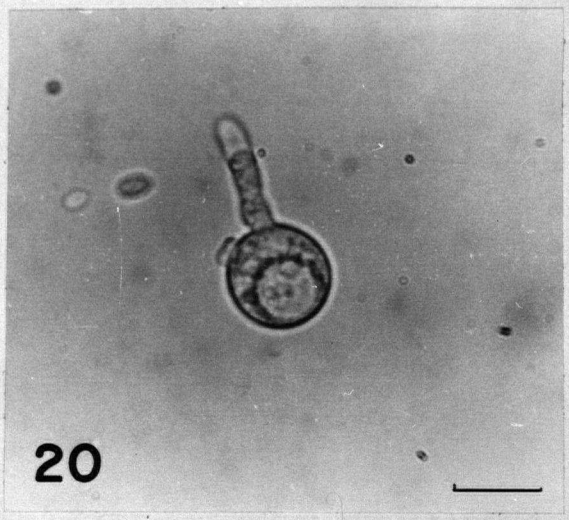
Fig. 20. Direct formation of a conjugation tube by basidiospore.

Fig. 21. Formation of a secondary spore by repetition.

Fig. 22. Basidiospore with sterigma.

Fig. 23. Formation of buds from basidiospore extension.

Fig. 24. Formation of terminal and lateral buds from aborted conjugation tube or sterigma.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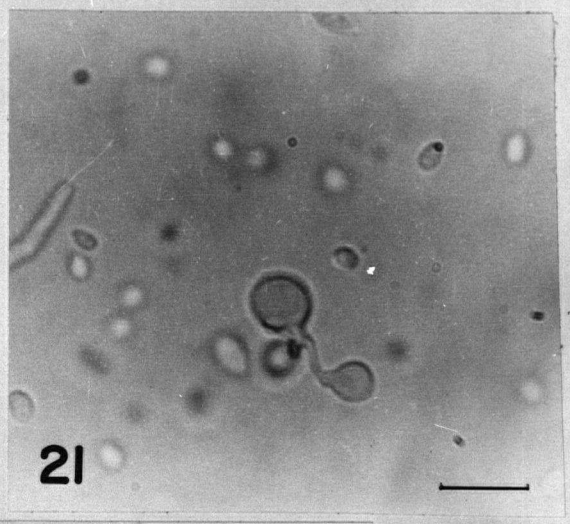
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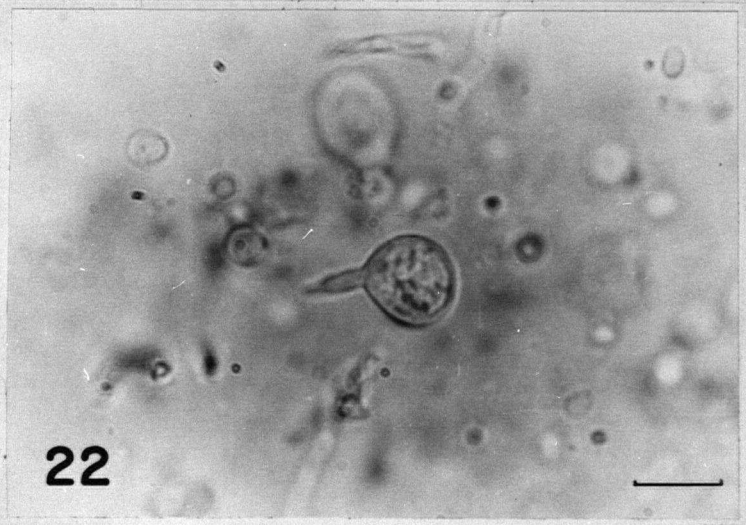
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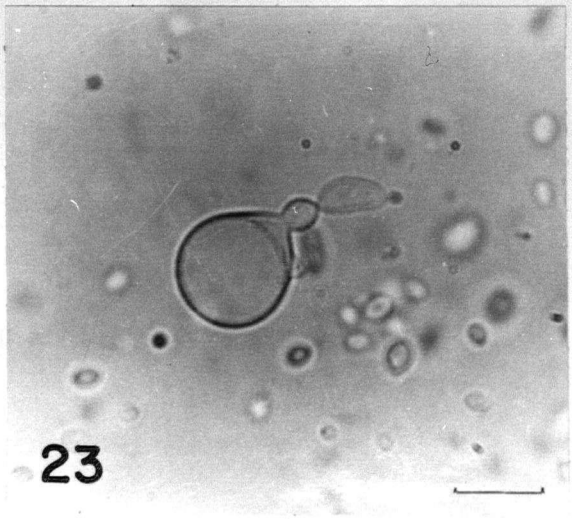
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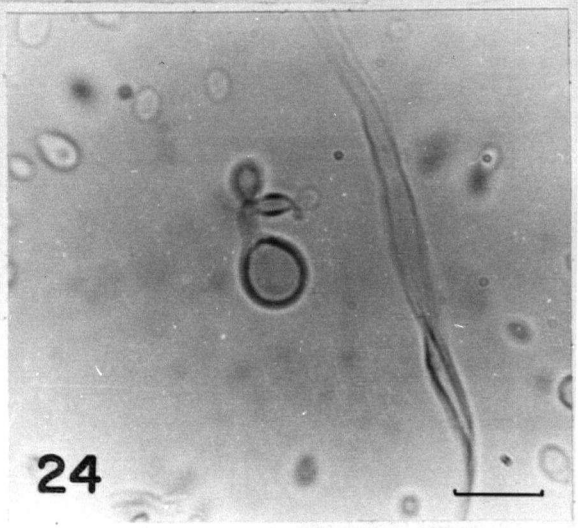
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B. Characteristics of the yeast phase

Colonies from single spores inoculated on MYS medium, are illustrated in Fig. 25. The cells multiply rapidly for the first week, forming a flat, sordid whitish colony, round in outline and with a smooth, shiny surface. The consistency varied with age and culture conditions but was mucous-like and either stiff or sticky. Older cultures often became distinctly pink or yellow and short hyphal strands were observed upon the agar surface. Hyphae did not reappear when the colony was transfered to new media. The colony produces no distinctive odor when grown on agar surfaces. In liquid medium, a slightly developed pellicle may form. This haploid phase was the subject of a series of nutritional and environmental experiments reported in the following pages.

C. Media

Calcium effects: Three randomly selected strains of *T. bambusina* were inoculated in the chemically defined medium SMA (See Appendix B: Media). After 4 days incubation at room temperature, only a slight indication of growth, if any, occurred in most cultures. No quantitative measurement of the growth was attempted. A series of medium modifications was made by substituting different carbon sources (galactose, xylose, sucrose, mannose) for dextrose and by adding different nitrogen sources, [$(NH_4)_2CO_4$, ammonium carbonate; $Ca(NO_3)_2 \cdot 4H_2O$, calcium nitrate] in addition to the asparagine. Additional vitamins also were added. This preliminary investi-

Figures 25 - 28

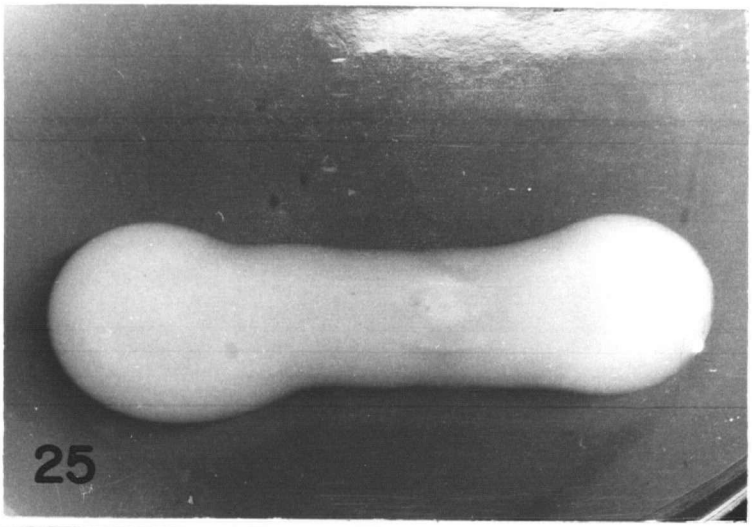
Growth of haploid phase.

Fig. 25. Appearance of colony growing on MYS medium. Seven days, at 20° C.

Fig. 26. Growth in modified SMC containing varying concentrations of $Ca(NO_3)_2$. Seven days, at room temperature.

Fig. 27. Growth in modified SMC containing different nitrogen sources. Seven days, at room temperature.

Fig. 28. Growth in modified SMC containing various vitamins. Four days, at room temperature.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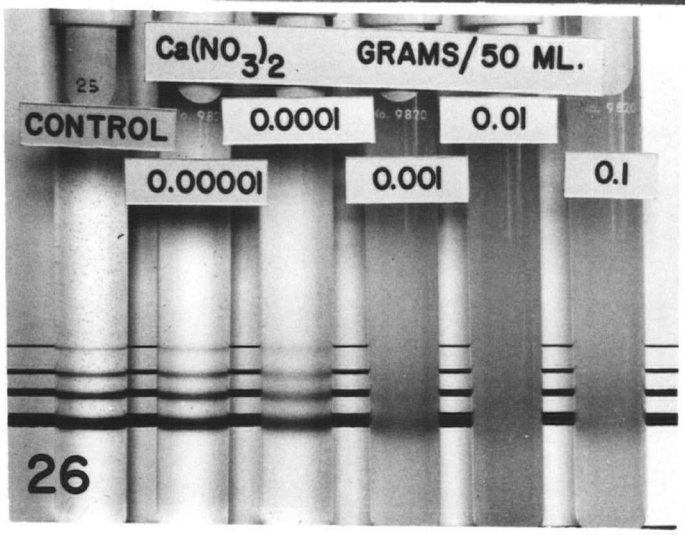
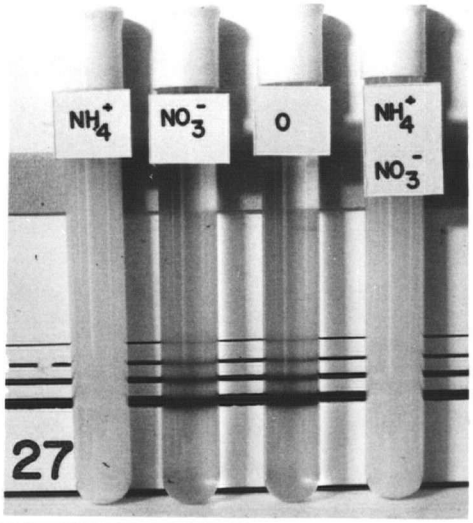
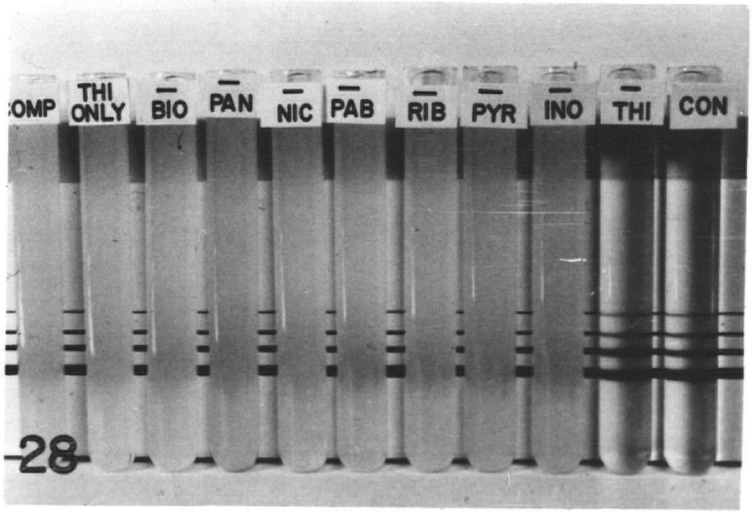

$Ca(NO_3)_2$ GRAMS/50 ML.
CONTROL
0.0001
0.01
0.00001
0.001
0.1
26


NH_4^+
NO_3^-
0
NH_4^+
NO_3^-
27


COMP
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28

gation showed substantial cell growth only where $Ca(NO_3)_2$ was added to the medium. Additional preliminary trials were prepared in an attempt to determine what component of the $Ca(NO_3)_2$ compound was influencing growth rate. Flasks were prepared with SMA as a control and the same medium with one of the following additions; $Ca(NO_3)_2 \cdot 4H_2O$; $CaSO_4 \cdot 2H_2O$, calcium sulfate; $(NH_4)_2SO_4$, ammonium sulfate; NH_4NO_3, ammonium nitrate; or KNO_3, potassium nitrate. In all flasks containing a calcium salt, a substantial increase in the growth of *T. bambusina* resulted. Only very slight growth occurred in flasks containing the ammonium salts or KNO_3.

Because of the current interest in the role of calcium in the growth of fungi, attempts were made to make the experiment quantitative and to provide closer control over the variables.

From parallel experiments to be described later, ammonium compounds were found to be good nitrogen sources for rapid growth and $(NH_4)_2SO_4$ was used as a substitute for l-asparagine of medium SMA. In addition $CaSO_4$ was added; this medium named SMB (See Appendix B: Media).

Eight strains of *T. bambusina,* representing two collections from *Thuja* (11, 12, 18 and 15, 17) and one collection from *Rubus* (522, 523 and 524) (all associated with *Valsa)* were grown in SMB. The eight strains were of different mating types, except 523 and 524, which were of identical mating type. The results indicated in Table IX show an

Table IX. Growth of *T. bambusina* on SMB with or without $CaSO_4$.

Incubation: Five days at room temperature.

Initial pH: 3.5

Inoculum: Identical measures in all flasks of one strain.

Replicates: Two.

	Calcium added			No calcium added		
11	1.9	(1.9-1.9)	2.4	0.013	(0.013-0.013)	3.35
12	0.74	(0.82-0.66)	3.0	0.004	(0.004-0.004)	3.45
15	1.9	(1.9-1.9)	2.5	0.056	(0.056-0.056)	3.4
17	0.401	(0.602-0.201)	3.4	0.022	(0.022-0.022)	3.3
18	0.008	(0.009-0.006)	3.45	0.008	(0.009-0.006)	3.4
522	0.469	(0.678-0.260)	3.0	0.004	(0.004-0.004)	3.4
523	1.0	(1.9-0.161)	3.0	0.006	(0.009-0.004)	3.4
524	0.053	(0.060-0.046)	3.4	0.032	(0.032-0.032)	3.4
Strain #	O.D.	Range	pH	O.D.	Range	pH

increase in cells resulted when $CaSO_4$ was added to the medium. Insignificant increases among two strains (18, 524) in SMB could be attributed to the character of the strains used.

Tests were made to determine if changes in calcium concentration influenced the optical density of the medium. $CaSO_4$ and $Ca(NO_3)_2$ were added to SMB in the following amounts, 0.4, 1.2 and 4.0 grams per liter. No detectable change in optical density of the medium was noted.

In an effort to increase the initial pH of SMB, I replaced $(NH_4)_2SO_4$ and $CaSO_4$ with $(NH_4)_2C_4H_4O_6$ (ammonium tartrate) and $Ca(NO_3)_2$ respectively, and designated this new medium as SMC (See Appendix B: Media). Five strains were selected, representing the complete range of variation from the first experiments. Attempts to show a correlation of growth response to increased amounts of $Ca(NO_3)_2$ were carried out and results are shown in Table X. Changes in measured growth among replicates of one strain were partially due to the varying temperatures in the flasks situated above the shaker motor. Variance of as much as 5° C. was possible from one area of the shaker deck to another and indicated the need for a constant temperature chamber for future growth studies.

The tolerance of strain 523 to 20 grams of $Ca(NO_3)_2$ per liter seemed to indicate that the role of calcium may be other than one of nutrition. Experiments not included in this thesis, involved substitution of equivalent amounts of $Sr(NO_3)_2$ (strontium nitrate) in place of $Ca(NO_3)_2$. The

Table X. Growth of *T. bambusina* on SMC with varying concentrations of $Ca(NO_3)_2$.

Incubation: Seven days, room temperature.
Initial pH: 4.8
Inoculum: Identical measures in all flasks of one strain.
Replicates: Two.

$Ca(NO_3)_2$ added Grams/liter	Optical density (Hi-lo readings)				
0	0.006 (0.007-0.004)	0.002 (0.002-0.002)	0.084 (0.097-0.071)	0.033 (0.034-0.032)	0.022 (0.022-0.022)
0.0002	0.020 (0.027-0.013)	0.028 (0.027-0.028)	0.114 (0.120-0.108)	0.054 (0.056-0.051)	0.024 (0.027-0.022)
0.002	0.056 (0.071-0.041)	0.215 (0.244-0.187)	0.272 (0.284-0.260)	0.490 (0.523-0.456)	0.032 (0.032-0.032)
0.02	0.084 (0.097-0.071)	0.52 (0.524-0.524)	0.86 (0.886-0.824)	1.0 (1.09 -1.0)	0.282 (0.456-0.108)
0.2	0.149 (0.161-0.137)	0.64 (0.68 -0.60)	1.1 (1.2 -1.0)	1.1 (1.2 -1.0)	1.2 (1.2 -1.2)
2.0	0.233 (0.398-0.066)	0.016 (0.018-0.013)	1.3 (1.2 -1.2)	1.3 (1.3 -1.2)	1.4 (1.5 -1.2)
20.0	0.011 (0.018-0.004)	contaminated	0.084 (0.097-0.071)	1.0 (1.0 -1.0)	0.056 (0.051-0.060)
Strain #	3	18	522	523	524

results were similar to those in Table X. Strains 522 and 524 which previously had shown little or no response to added calcium, showed a definite increase of growth with increasing concentrations of $Ca(NO_3)_2$. Microscopically the cells from all flasks, including those without a calcium salt appeared capable of further growth and development. This was verified with the subsequent increase of growth when $CaSO_4$ was added to the control medium at the end of the growth period, or when a portion of the control medium was inoculated into medium containing a calcium salt. The final optical density of each culture varied, for each $Ca(NO_3)_2$ level, possibly because of the concentration of cells in the initial inocula. Appearance of one set of samples after measurement for optical density, is shown in Fig. 26.

As additional experiments with calcium were performed, the results often were inconsistent with the findings previously made and the amount of growth between replicates also varied. This possibly could be attributed to temperature variation, light conditions, purity of chemicals or water, contamination of flasks, or strain type. Variation continued despite additional care and consideration of the above factors. Not until the following additional improvements of techniques and medium were used, were consistant results obtained. Closer tolerances in concentration of cells contained within the initial inoculum were obtained by standardizing to four drops inoculum from a 5 ml. source having a measured

optical density of 0.1. A constant temperature shaker-incubator was acquired and experiments were then carried out at a temperature of 20° C. A growth curve at this temperature is recorded in Graph 3 (See Appendix D). The ideal time for the measurement of final growth under the above conditions, is approximately 3 days after inoculation.

The number of ingredients of the medium was reduced to decrease the chance of contamination by chemical impurities. This was accomplished by using a four-element micro-element solution (See Appendix B: Media) rather than the more complicated Vogel's trace element solution. This medium was indicated as SMD (See Appendix B: Media). What appeared to be most critical, was the temperature of the inoculum and medium. Cells were suspended in calcium deficient SMD at 20°C., then inoculated into the medium at the same temperature. Results using these modified methods are recorded in Table XI, and indicate no change in growth rate in media lacking calcium, over a calcium enriched medium.

Nitrogen requirements: In preliminary experiments not included here, $Ca(NO_3)_2$ did not appear to serve as a good nitrogen source, but $(NH_4)_2SO_4$ and asparagine were capable of providing nitrogen for growth in liquid culture (Fig. 27). Table XII presents results confirming the failure of nitrate to be utilized. Ammonium compounds were a suitable source of nitrogen for the growth of *T. bambusina* . Asparagine, $(NH_4)_2$-SO_4 and $(NH_4)_2C_4H_4O_6$ both resulted in rapid growth of the

Table XI. Growth of *T. bambusina* on SMD with or without $Ca(NO_3)_2$

Incubation: Four days, 18° C.
Initial pH: 5.4
Inoculum: Two drops, prepared at 20° C. and introduced in medium at the same temp.
Replicates: Four

Strain number	Optical density (Hi-lo readings)	
18	1.5 (1.5 - 1.5)	1.5 (1.6 - 1.4)
12	1.6 (1.8 - 1.5)	1.5 (1.5 - 1.5)
11	1.5 (1.5 - 1.5)	1.5 (1.5 - 1.5)
8	1.6 (1.7 - 1.5)	1.5 (1.5 - 1.4)
523	1.5 (1.5 - 1.5)	1.5 (1.5 - 1.5)
522	1.5 (1.5 - 1.4)	1.5 (1.5 - 1.4)
527	1.4 (1.4 - 1.4)	1.5 (1.5 - 1.4)
611	1.5 (1.5 - 1.5)	1.5 (1.6 - 1.4)
612	1.5 (1.5 - 1.5)	1.5 (1.5 - 1.3)
613	1.5 (1.5 - 1.4)	1.4 (1.4 - 1.4)
618	1.5 (1.5 - 1.5)	1.5 (1.5 - 1.4)
710	1.5 (1.5 - 1.5)	1.5 (1.5 - 1.5)
712	1.4 (1.5 - 1.4)	1.5 (1.5 - 1.4)
701	1.5 (1.5 - 1.4)	1.4 (1.4 - 1.4)
711	1.5 (1.5 - 1.5)	1.5 (1.5 - 1.5)
	$Ca(NO_3)_2$ added	No $Ca(NO_3)_2$

Final pH = 2.7

Table XII. Growth of *T. bambusina* on modified SMD, using different nitrogen sources.

Incubation: Four days, 18° C.
Inoculum: 711
Replicates: Two

Nitrogen source	Optical density	pH At beginning	pH After 4 days
l-(+) Asparagine	1.5 (1.5-1.4)	4.6	4.2
$(NH_4)_2SO_4$	1.5 (1.6-1.5)	4.6	2.2
$(NH_4)_2C_4H_4O_6$	1.75 (1.7-1.8)	5.4	2.7
KNO_3	0.02 (0.02-0.02)	4.5	4.1
$Ca(NO_3)_2 \cdot 4H_2O$	0.01 (0.01-0.01)	4.3	4.2
None (control)	0.02 (0.03-0.02)	4.6	4.4

Table XIII. Growth of *T. bambusina* on modified SMD, using different carbon sources.

Incubation: Four days, 18° C.
Inoculum: 711
Replicates: Two

Carbon source	Optical density	pH At beginning	pH After 4 days
Glucose	1.7 (1.8-1.7)	5.4	2.7
Sucrose	1.6 (1.6-1.6)	5.4	3.1
Maltose	1.45 (1.5-1.4)	5.4	3.5
$NaC_2H_3O_2 \cdot 3H_2O$	0.01 (0.01-0.01)	6.3	6.2
None (control)	0.02 (0.02-0.02)	6.4	6.3

organism. Good growth was subsequently obtained when other ammonium salts were used. However, in instances where CH_3COONH_4 (ammonium acetate) and $(NH_4)_2C_2O_4 \cdot H_2O$ (ammonium oxalate) were used, the pH of the medium was high and a precipitate occurred during preparation. Medium SMD with $(NH_4)_2C_4H_4O_6$ as a nitrogen source, twice precipitated slightly upon autoclaving, but this redissolved after cooling. SMD containing $(NH_4)_2C_4H_4O_6$ as the nitrogen source, resulted in a higher initial pH value and an increased growth rate (Table XII).

Carbon requirements: The carbon compounds recorded in Table XIII were used as the sole source of carbon in carbon-modified SMD. The greatest measurement of growth, after 4 days incubation, took place where glucose was used. Good growth also was recorded, but at slightly decreasing levels, when sucrose or maltose were substituted for glucose. Sodium acetate did not support good growth. From microscopic observations, there was moderate growth in SMD where starch was employed as a carbon source, but the optical density was not recorded because of the heavy suspension of starch. Continued growth took place in all flasks except those containing either no carbon or $NaC_2H_3O_2$ as the carbon sources.

Vitamin requirements: Preliminary experiments on vitamin requirements using strain 523 and 12, and a vitamin modified SMC, are recorded in Table XIV. SMC, with thiamine only, consistently gave the greatest measured amount of growth.

Table XIV: Growth of *T. bambusina* on SMC modified with different vitamin sources.

Incubation: As indicated, at room temperature
Initial pH: 4.3
Inoculum: Identical in any series of one strain
Replicates: Five

Vitamins added	Optical density		
	Strain 523	Strain 12	Strain 12
Thiamine-HCl	0.88 (1.0 -0.74)	0.218 (0.356-0.114)	0.144 (0.265-0.050)
Biotin	0.130 (0.164-0.071)	0.041 (0.060-0.022)	0.050 (0.051-0.046)
Inositol and Biotin	0.097 (0.131-0.071)	0.036 (0.046-0.022)	0.051 (0.060-0.041)
Pyridoxine	0.071 (0.137-0.085)	0.041 (0.066-0.016)	0.075 (0.076-0.071)
Thiamine and Biotin	0.620 (0.92 -0.356)		0.337 (0.456-0.237)
Thiamine, Biotin, Inositol, Pyridoxine	0.52 (0.53 -0.495)	0.377 (0.398-0.356)	0.276 (0.272-0.284)
No vitamins added	0.086 (0.097-0.066)	0.071 (0.071-0.071)	0.065 (0.071-0.056)
Incubation	4 days	3 days	3 days

Thiamine appeared to be somewhat less effective in promoting growth when autoclaved in the medium at 121° C. for 20 minutes at pH 4.3. Additional expanded experiments were conducted in an attempt to produce more consistent and meaningful results. The results of these experiments, illustrated in Fig. 28, and tabulated in Table XV, agree well with the previous observations concerning the thiamine requirement. The results recorded in Tables XIV and XV were carried out before standard techniques of inoculation and growth were worked out. Therefore the range of recorded measurements of optical density between replicates, sometimes appears great.

pH requirements: The initial pH levels, of CJL are shown in Graph 1; after 3 days growth the optical density at each pH level listed, was measured and recorded. Final pH readings were not recorded for strain 12 or 710, but for strain 523 the final pH of the medium at the time of measurement, changed as follows; pH 4.0, +0.5; 4.5, +0.3; 5.0, -0.1; 5.5, no change; 6.0, -0.3; 6.5, +0.2; 7.0, no change; 7.5, -0.3; and 8.0, -0.2. The initial amount of inoculum and the temperature were identical for any given strain in tests shown in Graph 1.

The recorded pH range for each strain represents one experiment and not concurrent experiments. Therefore the different levels in optical density between cultures of different strains, are due to differences in inoculum amounts. Slow growth has been recorded at pH 2.5. Optical density

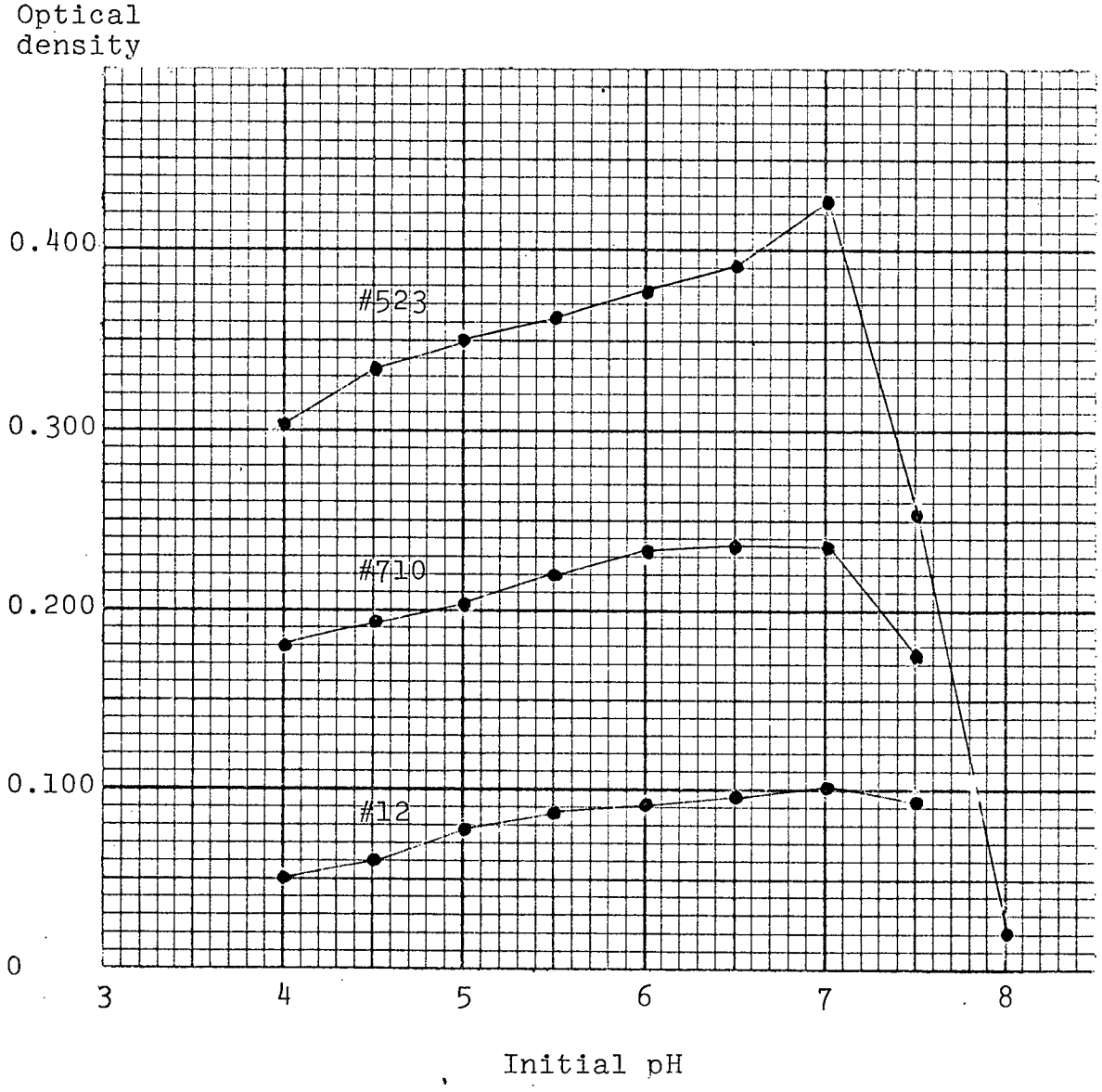
Table XV. Growth of *T. bambusina* on SMC modified with addition of different vitamin combinations.

Incubation: Four days, room temperature
Initial pH: 4.3
Inoculum: Identical measures for each strain
Replicates: Five

Vitamins added	Optical density	
Complete (all vitamins listed below)	1.4 (1.4 -1.4)	1.3 (1.4 -1.1)
minus Thiamine-HCl	0.161 (0.222-0.092)	0.119 (0.222-0.097)
minus Biotin	1.2 (1.3 -1.1)	1.1 (1.4 -1.0)
minus Riboflavin	1.2 (1.4 -0.79)	1.3 (1.4 -1.0)
minus Inositol	1.2 (1.4 -0.89)	1.3 (1.3 -0.75)
minus Nicotinic acid	1.4 (1.4 -1.1)	1.2 (1.3 -0.85)
minus p-aminobenzoic acid	1.2 (1.4 -0.82)	1.3 (1.4 -1.0)
minus Pyridoxine	1.2 (1.3 -1.1)	1.1 (1.2 -1.1)
minus Pantothenic acid	1.3 (1.4 -1.0)	1.3 (1.4 -1.2)
Thiamine only	1.5 (1.5 -1.4)	1.5 (1.6 -1.2)
no vitamins (control)	0.095 (0.119-0.081)	0.097 (0.108-0.071)
Strain #	523	12

Graph 1. Growth of *T. bambusina* in CJL at various pH levels.

Incubation: Three days, room temperature
Initial pH: As indicated.
Inoculum: Identical measures for each strain
Replicates: Two



of liquid CJL could have varied slightly through various pH levels. In view of this, other experiments were carried out in MYS at a constant temperature of 20° C. and are represented by the figures shown in Table XVI. The pH values near 7.0 were most suitable for the rapid growth of *T. bambusina*, but reasonable growth also occurred down to at least pH 4.0 (and from other observations down to pH 2.5). When all strains and the slight changes in pH that occurred during growth were compared, it appeared that by increasing the initial pH over the acid range, there was a subsequent increase in the measured rate of growth in liquid culture. The maximum growth rate appeared near pH 7.0. At levels above pH 7.0, the growth rate declined rapidly.

D. Conjugation phase

Characteristics: All basidiospores and buds have the potential for producing conjugation tubes if stimulated by the conjugation hormone from the opposite mating type. The ability of a cell to form a conjugation tube connot be predetermined by visual examination. I noted that not all the daughter cells from one spore or bud produced a conjugation tube even though all were present in what appeared to be identical environments (Fig. 29). On spore-drop plates, almost all daughter cells from certain basidiospores formed tubes while in other nearby clones, as few as one cell produced tubes.

Microscopic observations showed that the conjugation

Table XVI. Growth of *T. bambusina* on MYS at various pH levels.

Incubation: 20° C., 4 days
Inoculum: 1 drop of very dilute suspension in all flasks.

pH prior to inoculation	2	3	4	5	6	7	8	9
pH after growth	2.15	3.0	3.85	4.4	4.8	5.5	6.1	6.15
Number of cells per 0.004 cu. mm. (average of five counts)	1	128	259	880	960	1184	1026	1104

Figures 29 - 35

Formation of conjugation tubes on CJA. Photographed *in situ*.

Fig. 29. Initiation of a conjugation tube from one cell while other cells show only budding.

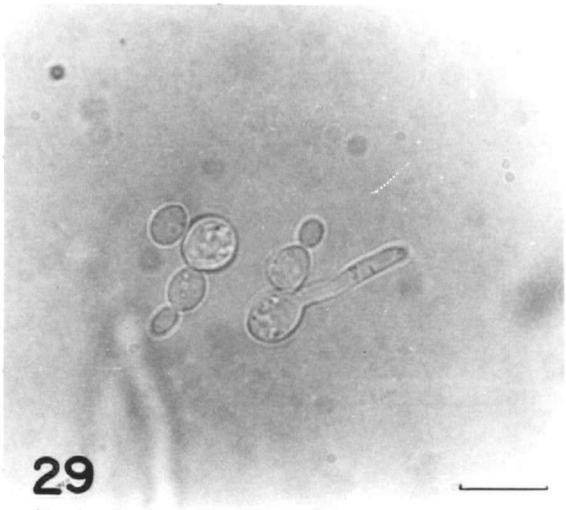
Fig. 30. Formation of septum (arrow) separating protoplasmic filled portion from empty portion and original cell. Tip has reverted to budding growth.

Fig. 31 and 32. Formation of a terminal cell and blastospore development from the latter and from tube apex.

Fig. 33. Formation of a lateral conidium on conjugation tube.

Fig. 34. Conjugation tubes from compatible cells showing tropism where tips have approached to within critical range of one another.

Fig. 35. The fusion and flattening of conjugation tube tips of a compatible pair.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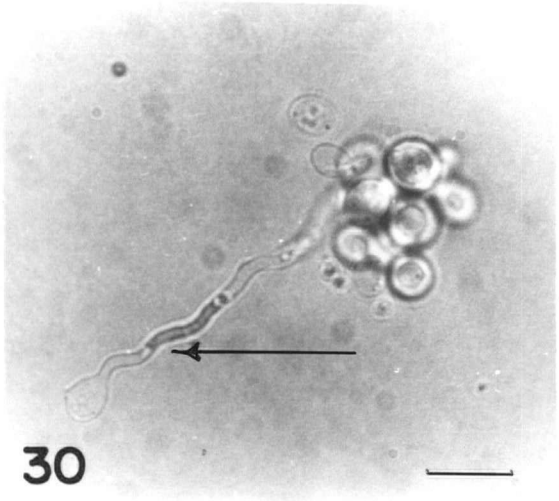
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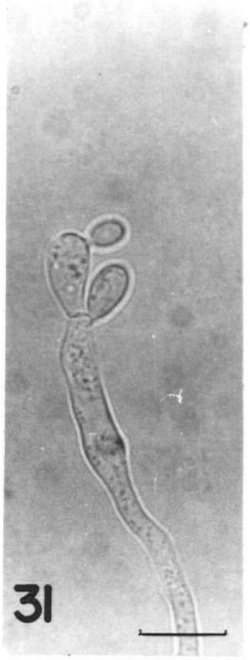
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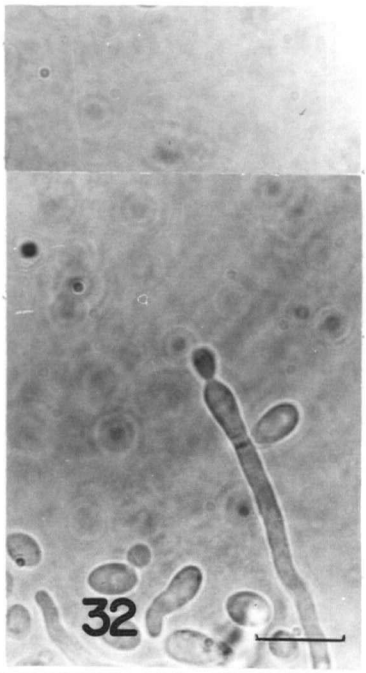
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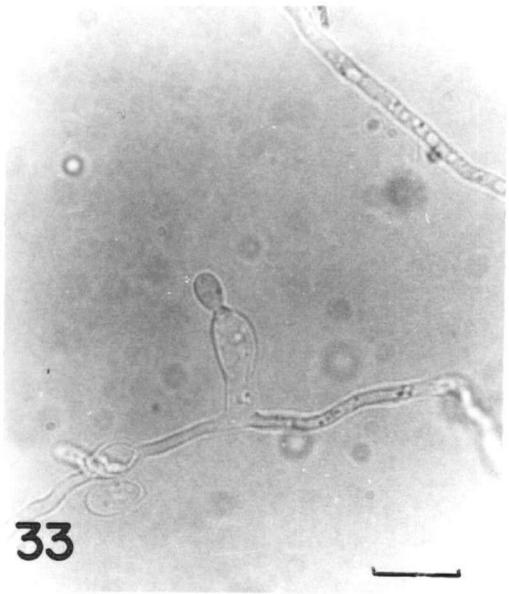
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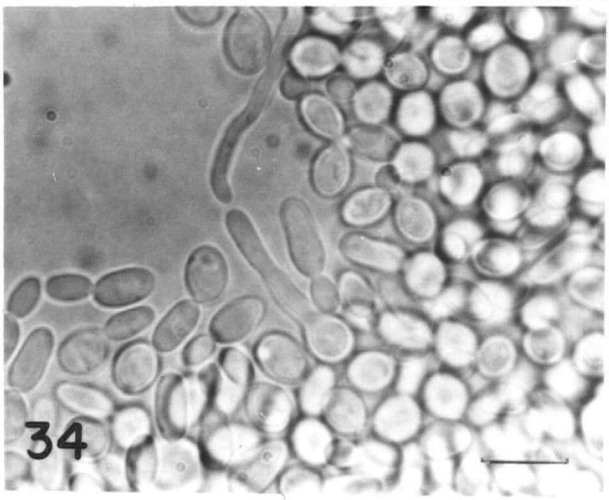
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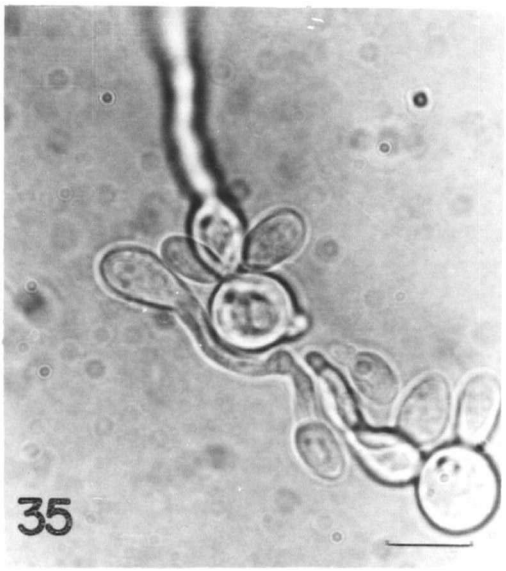
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tube began with the apparent failure of a bud to complete its development by pinching off. Instead the bleb continued to elongate and was capable of unlimited growth. Occasionally conjugation tubes appeared from opposite ends of the cell, or the tubes branched. Septa, formed back of the growing tip, appeared to separate the protoplasm containing portions of tubes from empty portions thereof (Fig. 30). During the first day of growth, hyphal strands increase terminally at the rate of about 5 μ. per hour at room temperature. My observations indicated that growth could continue at this rate for 3 or more days or it could suddenly cease. The growing tips of conjugation tubes were found to be sensitive to the concentrated light during microscopic examination. Illumination therefore was maintained at the lowest level allowing observation and for short periods of time only. In many instances a septum formed below the tube apex and the hyphae produced conidia terminally and subterminally as well (Fig. 31, 32). Conidia were infrequently produced laterally on the conjugation tube (Fig. 33). All conidia appeared to be capable of normal budding and conjugation, and resulted in cells indistinguishable from those arising from the germinating basidiospore or its daughter cells.

Conjugation tubes appeared to grow in a random direction except when the extremities of two germ tubes compatible for the "B" factor approached one another by chance. In instances where the tube tips were close enough to one another, and

were of compatible mating types, a mutual stimulating effect apparently caused the tips to grow toward one another (Fig. 34). End-to-end contact occurred, and the tubes flattened against one another prior to fusing (Fig.35). The tropic response was observed to occur over a range measured at up to 10 μ. If the tips were not within ca. 10 μ. of compatible tips, there was continued non-directional growth. After contact, the flattened tube tips lyse so that the two tubes became confluent and the dikaryotic phase was initiated. A dikaryotic hypha then grew from one of the two conjugating cells, if the tubes formed were short (Fig. 36). If the conjugation tubes were long before contact was made and septa had been laid down, the dikaryotic hyphae grew from near the area of contact. Clamp formation occurred within 24 hours after pairing, and the dikaryotic hyphae increased in length initially at a rate similar to that of conjugation tubes (5 μ. per hour).

Compatible pairs, previously shown to be excellent tube producers, were inoculated into liquid SMC, MYS and CJL. One loopful of inoculum was added directly to the medium. With these media, three methods of bringing together compatible types were employed. 1. Compatible strains were incubated at room temperature in liquid culture, shaken continuously for 3 days, then poured together and kept under similar conditions for 3 additional days; 2. Paired strains were incubated together at room temperature on the shaker for 10 days;

Figures 36 - 41

Dikaryotic hyphae formed on CJA. Photographed *in situ*.

Fig. 36. Formation of dikaryotic hypha from two compatible cells (arrows). The conjugation tubes fused somewhere between cells and a dikaryotic hypha grew from opposite end of cell 1 and formed the first clamp connection. Cell 2 had formed conjugation tubes from opposite end of the cell prior to actual conjugation.

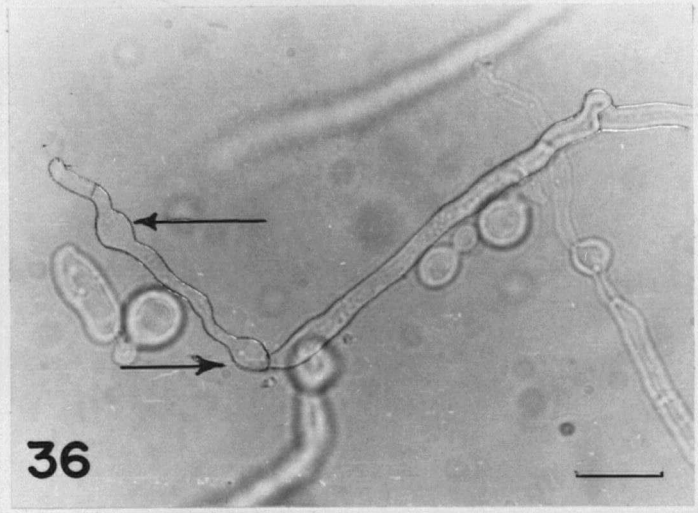
Fig. 37. Growing tip of dikaryotic hypha showing a single clamp connection and hyphal extension from clamp area.

Fig. 38. Clamped hyphal extension arising from main hyphal clamp.

Fig. 39. Dichotomous branching. One extension (arrow) formed from a clamp similar to that described in Fig. 37 above.

Fig. 40. Paired clamp extensions.

Fig. 41. Haustoria formed from area of clamp connection.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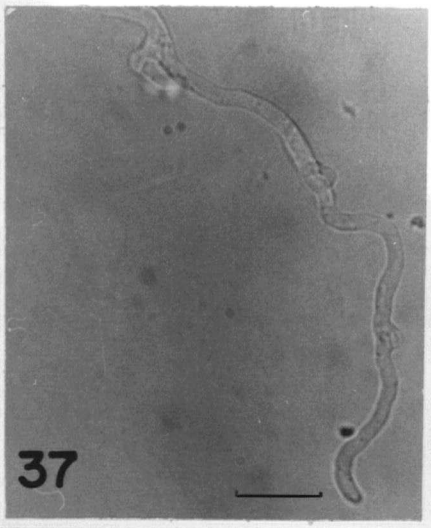
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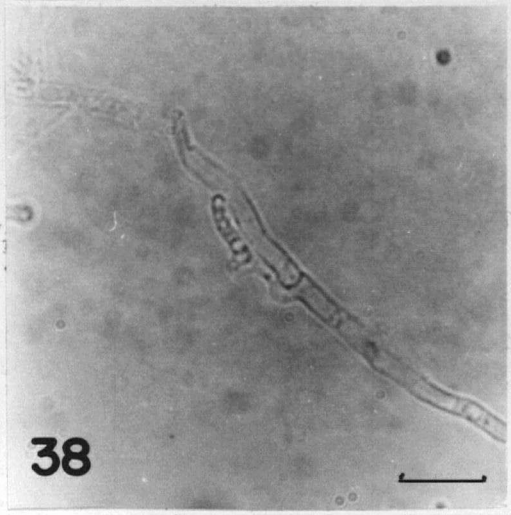
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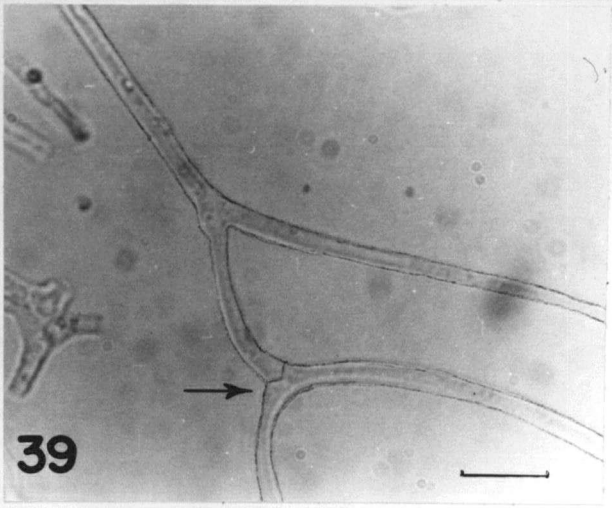
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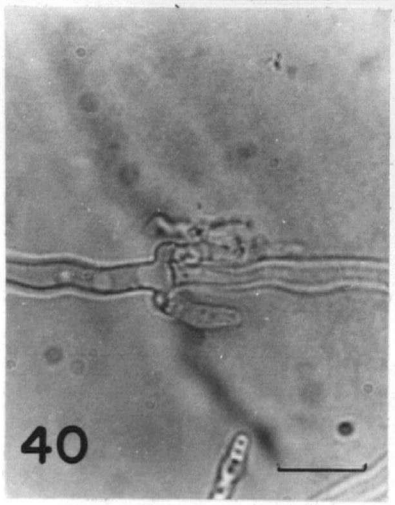
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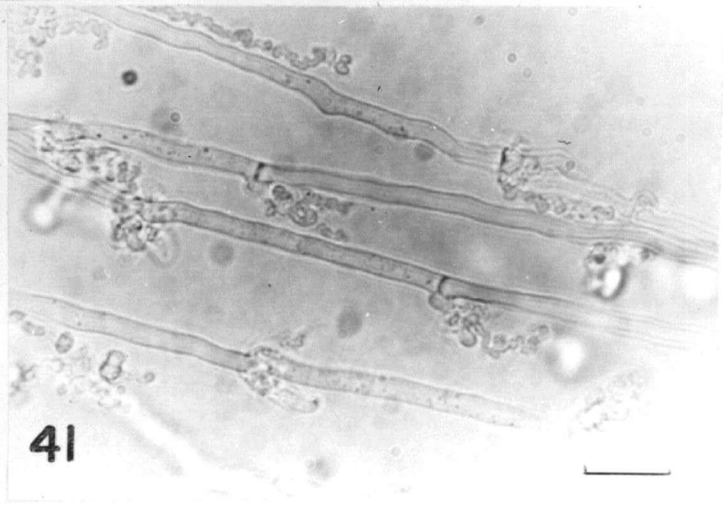
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and 3. The same conditions as "2" above, except after 6 days the cultures were placed in a 15° C. stationary incubator. Half of the cultures were kept in continuous darkness, (accomplished by wrapping flasks in aluminum foil). The remainder were not covered and were subjected to normal day-night periods of light and dark. Varying the strains, medium type, agitation period and light conditions failed to induce consistent tube initiation between compatible strains in liquid culture. A few conjugation tubes were always observed where compatible strains were grown together in liquid culture. However, the number of tubes was small, ranging from almost no tubes in 50 ml. of culture medium to perhaps less than one tube per 500 budding cells. When samples of the compatible pair, grown in liquid culture at 20° C., were deposited upon CJA or MYS medium, tubes were initiated, with CJA producing slightly better results than MYS. This ability was tested over a period of 10 days and in every test, conjugation tube formation occurred within 24 hours after deposition upon solid medium.

Variation in conjugation tube production from cross to cross and from plate to plate of the same cross was observed during the compatibility studies. Standardization of inoculation procedures appeared necessary before consistent and meaningful tests could be carried out on the ability of pairs to form conjugation tubes and to proceed in subsequent development. Data from Table XVII indicate that while some con-

Table XVII. Physical conditions affecting the initiation of conjugation tubes in *T. bambusina*.

Incubation: 18° C. for 2 or 4 days, unless otherwise specified.

Replicates: Five

Age of inoculum	Two days old Twenty days old	++++ +++
Amount of inoculum	Four loops Minimum amount	+ ++++
Amount of mixing	Minimum Maximum	++ ++++
O_2 - CO_2 concentration	Coverslip added to colony No coverslip added	++ ++++
Depth of medium	2 mm. 8 mm.	++++ +++
Light conditions	Dark Light	++++ ++++
Temperature	18° C. Room temperature	++++ ++++
pH	6.0 3.0 or 8.0	++++ ++
Age of medium	1 day old 30 day old	++++ ++++

Key ++++ = numerous tubes, approx. 150+/100 $\mu.^3$ of medium.
+++ = many tubes, approx. 50-100/100 $\mu.^3$ of medium
++ = few tubes, approx. 10-30/100 $\mu.^3$ of medium.
+ = tubes rare, less than 5/100 $\mu.^3$ of medium.

ditions resulted in slight differences in number of conjugation tubes, certain combinations might show clearly detectable differences. As a result of these studies, experiments on the ability of certain stocks to initiate basidia, produce basidiospores or form basidiocarps, were carried out. Standardized methods of inoculation and medium preparation developed and recorded in the methods section for the compatibility studies were used.

Possible production of autoantibiotic substances was tested when compatible strains were streaked on the surface of CJA or MYS and incubated at 18° C. for 7 days. Conjugation tubes were formed, but only along the outer margins was abundant dikaryotic mycelium produced. The inner streaks showed a definite inhibition of mycelial development and growth (Fig. 42). Growth of the yeast phase appeared to be uninhibited. I observed also that plates containing a "lawn" of compatible cell pairs also produced weak conjugation tube growth and dikaryotic hyphal growth.

E. Conjugation hormones

To determine if conjugation hormones of *T. bambusina* behaved as described in other species of *Tremella* (Bandoni, 1965, Flegel, 1968), similar experiments were performed.

The experiments, attempting to show that the conjugation hormone(s) were capable of diffusing through dialysis membranes, were positive. Observations on six compatible pairs, showed in all instances, the formation of conjugation tubes

Figures 42 - 45

Effects of inhibitory substances.

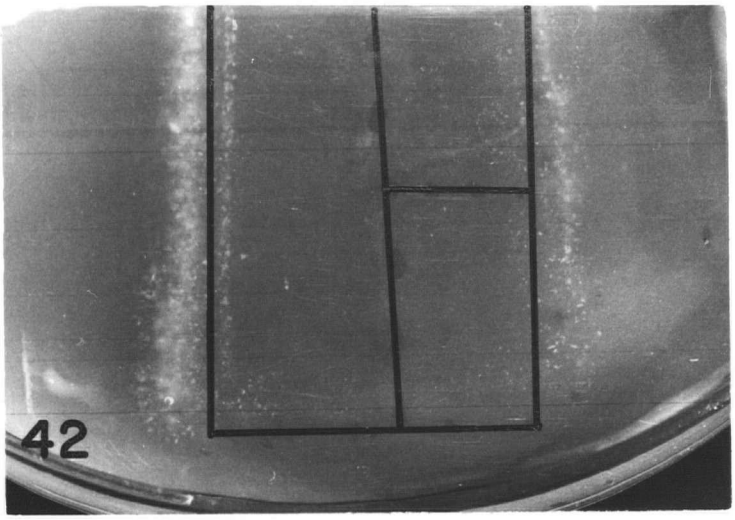
Fig. 42. MYS medium which had been inoculated with compatible haploid strains in the areas indicated by the inscribed lines. The haploid cells were removed to show the pattern of formation of abundant dikaryotic growth along the outer streaks. Very little visible growth occurred from the inner streaks.

Growth of the dikaryotic phase. Photographed *in situ*.

Fig. 43. Growth of dikaryotic mycelium on PSA, 1 month at 15° C. under alternating 12-hour periods of dark and light, showing formation of gelatinous basidiocarps.

Fig. 44. Growth of dikaryotic mycelium on MYS 1 month at 15° C. under alternating 12-hour periods of dark and light, showing formation of non-gelatinous, recurved, fibrillose scales.

Fig. 45. Growth of mycelium on PSA, 1 month at 10° C. in complete absence of light, showing matted mycelial characteristic.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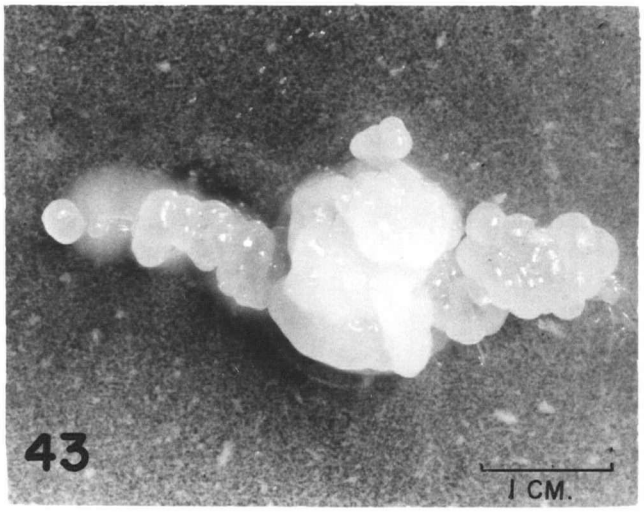
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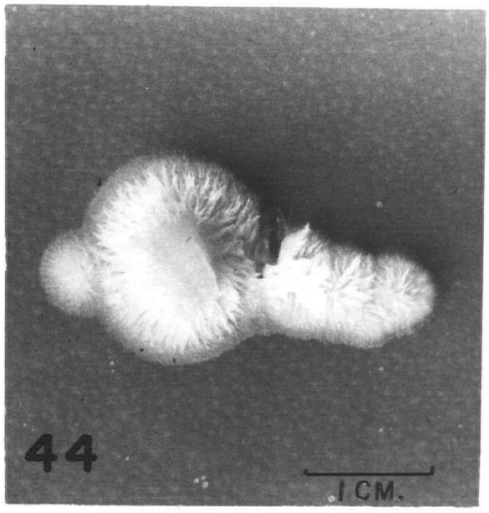
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1 CM.


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by strains on both sides of the membrane, with greater numbers of tubes formed by the strain below the membrane. The continuous production of conjugation hormone(s) was indicated when tubes were present in an agar area where compatible cells had previously been grown on dialysis membrane and removed. No dikaryotic hyphae were observed. There was a direct correlation between the length of time hormones were produced and the length of the conjugation tubes. The longest tubes grew where the compatible cells had been growing for 6 days prior to their removal. Shortest tubes were found after the membrane was removed with a 2 day-old colony of compatible cells.

The hormones controlling initial tube formation were measured at the end of 5 days to have been effective over a lateral distance of at least 20 mm. of CJA at 18° C. The number of tubes generally diminished the further apart the two strains were grown.

No conjugation tubes were observed after a strain was allowed to grow for 4 or 7 days on CJA or MYS medium, autoclaved, then the same area reinoculated with a non-autoclaved compatible strain. The viable strain grew rapidly indicating the condition of the medium was still capable of supporting good growth of the yeast phase, but no tubes were initiated. As a check to insure that the media were still capable of supporting tube initiation and growth, inoculation of the first strain in viable form was again made and resulted in

the formation of conjugation tubes between the viable pair. These results were obtained with compatible pairs 711 X 8, 544 X 548, and 522 X 2. An additional experiment was devised to take advantage of quantities of hormone presumably produced by strain 523 grown in liquid SMC culture for 8 days until maximum growth was obtained. A filtrate of this was used to make up 500 mls. of solid medium to which 1.0 g. soytone and 1.0 g. dextrose was added along with the 10 g. of agar. This medium was divided and adjusted to 7 pH values by the addition of 0.25 N NaOH or 1 N HCl, autoclaved at 121° C. for 15 minutes and poured into Petri plates. Five replicates were inoculated with strain 522, then incubated at 15° C. No conjugation tubes were observed at any of the seven levels from pH 3.0 to pH 9.0, although growth and continued budding of the viable strain was observed at all levels except pH 9.0.

III. Dikaryotic phase

A. Mycelium development

Once the dikaryotic phase had been established, it grew relatively slowly on laboratory media and usually was overrun by the more rapidly growing haploid phase. In week-old cultures of paired strains, single dikaryotic hyphae could frequently be observed along the edge of the haploid phase. With the aid of a dissecting microscope, single strands of these hyphae could be transferred separately to new medium. Alternatively, the dikaryotic mycelium could be obtained by scraping off the yeast phase with a sterile coverslip, then

covering the colony with a sterile agar block cut from the edge of the medium. The mycelium grew up through this agar and could easily be transferred when it reached the surface. The entire process from pairing to transfer could be accomplished in less than 10 days. Once established, the dikaryotic hyphae appeared to be capable of unlimited growth and was easily distinguished, microscopically, from conjugation tubes, by the presence of clamp connections.

Simple clamp connections were formed early in the growth of dikaryotic hyphae (Fig. 36) and were abundant in older established colonies and developing basidiocarps. Frequently a hyphal extension would develop from the clamp (Fig. 37) forming a major branch (Fig. 39). These hyphal extensions most often develop a true clamp close to the emergence point (Fig. 38) but thinner hyphae may be formed (Dia. 1; k) and become knotted (Fig. 41). These hyphae I termed haustoria because of the similarity to structures designated as haustoria by Olive (1946). Extensions were frequently seen in pairs, opposite each other, from septal areas (Fig. 40). Extensions also arose from what appeared to be completed clamps. In all instances where the dikaryotic phase continued to grow, the yeast phase eventually became evident. Although often not visible macroscopically, haploid cells could always be demonstrated microscopically and often overran the mycelial phase if additional transfers were not made. Neither the media nor the physical conditions employed were

sufficient to maintain a strictly dikaryotic phase over a period of more than 2 weeks, although low temperatures appeared to restrict rapid yeast growth. The haploid phase in most instances was the result of blastospore production by hyphae, although the germination of basidiospores might have been responsible in part. Attempts to maintain a strictly dikaryotic phase in SMC, MYS or CJA liquid medium under varying conditions of temperature and light were never successful. Cultures inoculated with rapidly growing dikaryotic mycelium would dedikaryotize and an abundance of budding cells was produced.

Throughout the compatibility studies, I observed that there was extreme variability of numbers of conjugation tubes initiated and dikaryotic hyphae established. Continued observations, using a variety of media and conditions, indicated that certain crosses could be consistently classified as either strong or weak producers of dikaryotic hyphae. Although there was a complete gradation from weak to strong, certain strains were consistently involved in the extremes. When both compatible strains were classified as weak, few dikaryotic hyphae were established; where one or both strains were classified as strong, greater numbers of pairs conjugated. The characteristic variability of quantities of conjugation tubes formed between fully compatible pairs, appeared to be genetically controlled and thus influenced choice of strains for further study of the dikaryotic phase.

Dikaryotic mycelium resulting from the pair 523 X 2, was allowed to grow 1 month on MYS medium. As a measurement of growth, the diameter of each colony was recorded at 11 temperature levels. Those measurements above 20° C. are not significant because of the dehydration of the medium at these higher temperatures. The results are shown in Graph 2. It was established from additional observations that the dikaryotic phase grew slightly at the extremes of 3° and 28° C. The most growth of this phase appeared to be between 15° and and 25° C.

From the results recorded in Table XVIII, where various combinations of medium, temperature and light conditions were present, the dikaryotic stock 12 X 15 grew better on MYS and PDA than on CJA medium, and better at 15° than at 4° C. There was very little difference between the diameter of the colonies on MYS compared to those on PDA medium, but the texture of the colonies was noticeably different (Fig. 44-45). Even though the initial inoculum often was yellow, there was a change in the color of the colony to white as the dikaryotic phase developed at the 4° C. range. The presence or absence of light in these experiments appeared to make little difference in the degree of growth or the gross morphology of the dikaryotic colony.

B. Basidiocarp development

Because the dikaryotic mycelium in culture is capable of growing directly into gelatinous basidiocarps, the conditions

Table XVIII. The effects of medium, light and temperature on the growth of the dikaryotic phase of *T. bambusina*.

Replicates: Four

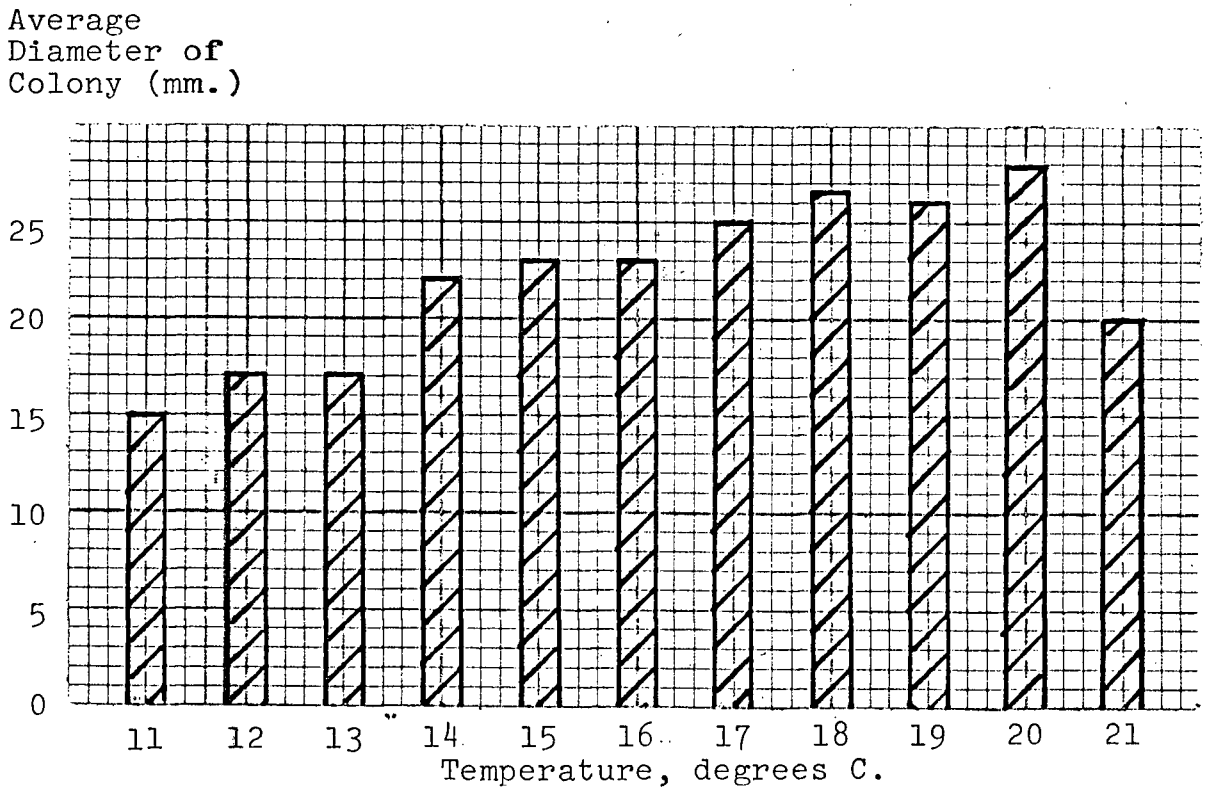
Medium	Light conditions	Temperature 4° C.	15° C.
MYS	Light	+	+++
MYS	Dark	+	+++
PDA	Light	+	+++
PDA	Dark	+	+++
CJA	Light	+	++
CJA	Dark	+	++

Key

+++=Rapid growth
++ =Slow growth

Graph 2. The effects of temperature on the diameter of dikaryotic colonies of *T. bambusina* grown on MYS.

Replicates: Four



influencing the establishment and growth of the mycelium also affected the formation of the basidiocarp. The degree to which the basidiocarp developed had little relation to the development of basidia and basidiospores. Well formed basidia, complete with septa, were frequently observed as the mycelium grew through the agar medium. What appeared to be typical gelatinous basidiocarps frequently possessed very few basidia.

One month after inoculation with stock 12 X 15, gelatinous basidiocarps were common on PSA medium at 15° C. (Fig. 43) but not on MYS medium at the same temperature (Fig. 44). When grown at 4° C. and 10° C. the same stock resulted only in mycelial colonies consisting of recurved or matted, fibrillose scales. The latter often extended up from the surface of the agar and were similar to the scales on the basidiocarp seen in Fig. 44. To determine if this consistency and texture was caused by nutrients in the medium or by conditions surrounding the developing basidiocarp (e.g. humidity, gas concentrations, condition of inoculum), a technique was developed that resulted in formation of numerous basidiocarps on one plate and from a single stock.

A developing basidiocarp or the surrounding mycelium within a plate was cut and a portion removed to new medium. Several shallow cuts were made by dragging the transfer needle through the inoculum and across the new medium surface. This distributed small portions of the mycelium to new locations across the medium. The mycelium developed rapidly

beneath the agar surface while the exposed portions of mycelium developed into basidiocarps. Within 10 days, numerous basidiocarps at various stages of development could be found on a single plate. Some dedikaryotization occurred, especially near the original inoculum and haploid cells quickly covered some basidiocarps. Well isolated basidiocarps, and those found at the edge of the cuts, on MYS, PDA and PSA, were characteristic of those found in nature. Basidiocarp surfaces ranged from smooth to fibrillose on any single plate, and were not consistent on any medium types or at any light or humidity conditions.

Stock 12 X 15 was also inoculated upon MYS containing actively growing mycelium of *Valsa* sp. or extracts from *Valsa* perithecia. No consistent, detectible differences were noted with respect to basidiocarp, basidia or basidiospore development on such media when compared with controls. This same stock was inoculated in various positions in respect to the growing mycelial colony of *Valsa*. *Valsa* hyphae grew rapidly and eventually overran the slow growing *Tremella*. I observed no visible interaction where the margins of each colony approached and grew past one another. After 3 weeks, mycelial growth of *T. bambusina* on medium containing sterilized *Valsa* perithecia did not appear to differ from that on MYS controls.

Sections of *Rubus spectabilis* cane or *Thuja plicata* branches were autoclaved in culture tubes to which 2 ml. of distilled water had been added. Agar blocks containing

mycelium of *Valsa* sp. or stocks of *T. bambusina* were deposited upon each substrate and incubated one year at 15° C. Both substrates, when inoculated with *Valsa* alone or *T. bambusina* alone, showed only continued hyphal growth in all replicates. *T. bambusina* basidiocarps were formed on agar blocks on both substrates where *Valsa* had been inoculated.

Stocks 543 X 2, and 711 X 8 were more consistent producers of functional basidia than the stock 12 X 15 previously used. These two new stocks were used for further studies on basidiocarp formation.

Basidiocarps formed by stocks 543 X 2 and 711 X 8 were cut into uniform blocks, inoculated upon MYS and allowed to grow under conditions described in the methods section. At the end of 10 days, conditions were changed (see Table XIX) and observations were made at the end of 5, 11 and 18 additional days growth. Three replicate cultures were grown under each set of conditions; only final measurements were included in the table. There was continued growth of the basidiocarp and the yellow appearance was retained under all conditions tested. The greatest increase in the size of the basidiocarps resulted where additions of asparagine, vitamins or $Ca(NO_3)_2$ were made. The largest basidiocarps formed where $Ca(NO_3)_2$ crystals were added, but the basidiocarp consistency was very rubbery. The consistency of the basidiocarp most closely approached that found in nature on medium with the added vitamins. Cultures incubated at 15° C. were subjected

Table XIX. Basidiocarp, basidia and basidiospore formation on MYS, at 15° C., under alternating 12-hour periods of light and dark.

After 10 days growth, conditions were changed by:	Average size of basidiocarp. (diameter X height)	Number basidia with septa.	Quantity of basidio-spores.
adding asparagine crystals	1.6 X 0.5 cm.	2 - 10%	1 - 10/slide
adding $Ca(NO_3)_2$ crystals	2.3 X 0.7 cm.	2 - 10%	1 - 10/slide
adding 1 ml. of four vitamin stock solut.	1.8 X 0.6 cm.	40 - 90%	1 - 10/slide
removing agar from around basidiocarp	0.6 X 0.5 cm.	1 - 2%	0
placing near window at room temperature	1.3 X 0.4 cm.	11 - 40%	10-50/slide
covered with foil at room temperature	1.8 X 0.6 cm.	0%	0
No changes (Control)	1.3 X 0.4 cm.	2 - 10%	1 -10/slide

to alternating 12-hour periods of light and dark, and the basidiocarps were yellow. Basidiocarps developed at 4° and 10° C. and retained in darkness, except for short observation periods, were white after the same length of time but eventually and slowly became yellow.

C. Basidium development

The first indication of basidial development resulted when the terminal cell above a clamp enlarged (Fig. 46) and stained deeper. As the basidium developed, a second basidium often developed from the clamp at the base of the first basidium, or more frequently, from the next clamp back (Fig. 47, 48). Typically, as the probasidium became septate (Fig. 49, 51), epibasidia developed from the apex (Fig. 50, 52, 54) and the cytoplasm became highly vacuolate. Under culture and natural conditions, the probasidium often became irregularly septate (Fig. 52, 53). It has not been ascertained what factors cause this irregular appearance. Observations in culture show that, although the epibasidia often developed normally, they sometimes became atypical by septation (Fig. 57), branching, or production of apical conidia (Fig. 56). Observations here and in the literature demonstrate non-synchronous development of each of the four cells of the probasidium. The epibasidia and metabasidia collapsed following spore discharge (Fig. 58) and indicated possible spore production.

There was extreme variability in the abundance of pro-

Figures 46 - 53

Formation of basidia in culture.

Fig. 46. Early stage in basidium formation showing the enlarged terminal cell of a hyphal strand and basal clamp.

Fig. 47. Terminal basidium with a lateral basidium developing from a lower clamp (arrow).

Fig. 48. Two developing basidia, the lateral one with a basal stalk. Both stalked and stalkless types occurred in culture.

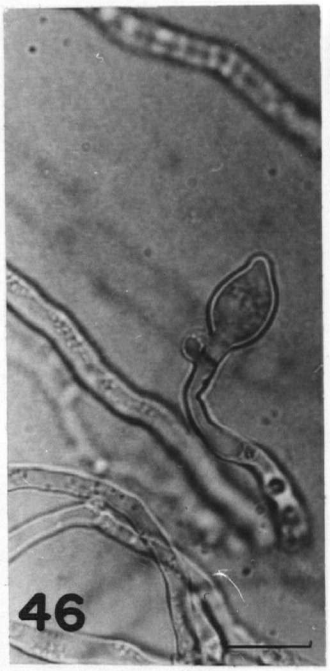
Fig. 49. A basidium with a typical, obliquely-developed septum.

Fig. 50. Initiation of an epibasidium from a metabasidium.

Fig. 51. Top view of a basidium showing cruciate septa.

Fig. 52. Two basidia, one showing the formation of an epibasidium (left), the other with irregularly formed septa (right).

Fig. 53. Empty, collapsed cells of metabasidium after spore discharge. One cell on right still filled with protoplasm. Epibasidia are not apparent from this view.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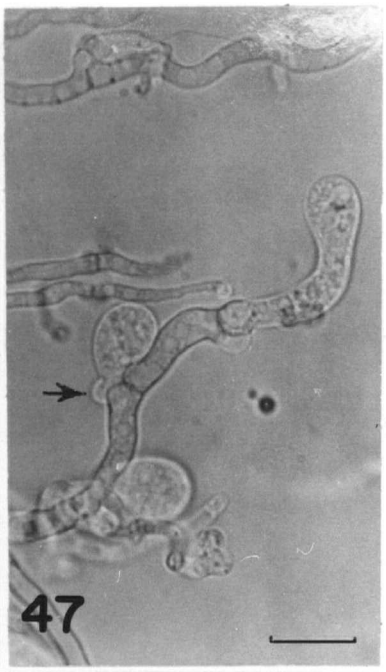
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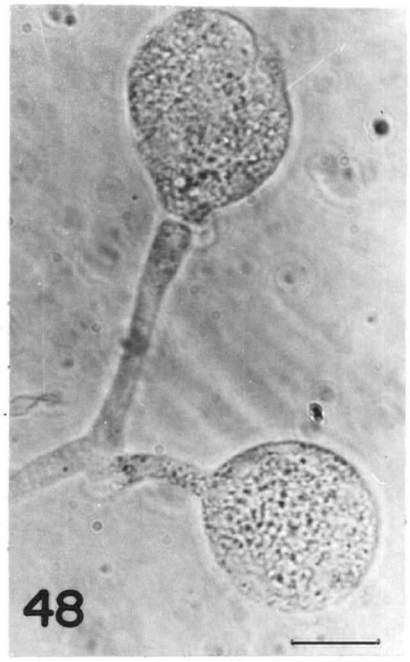
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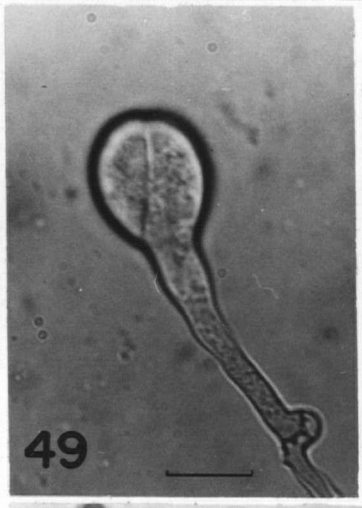
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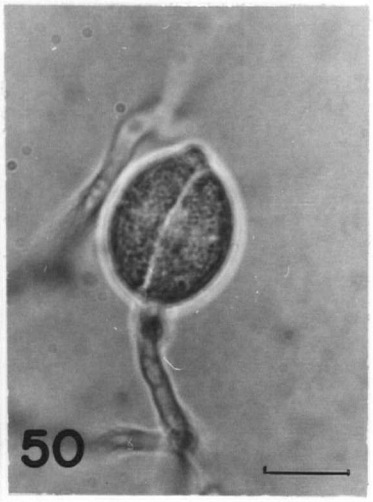
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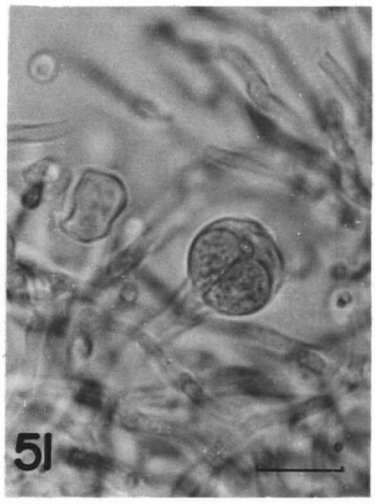
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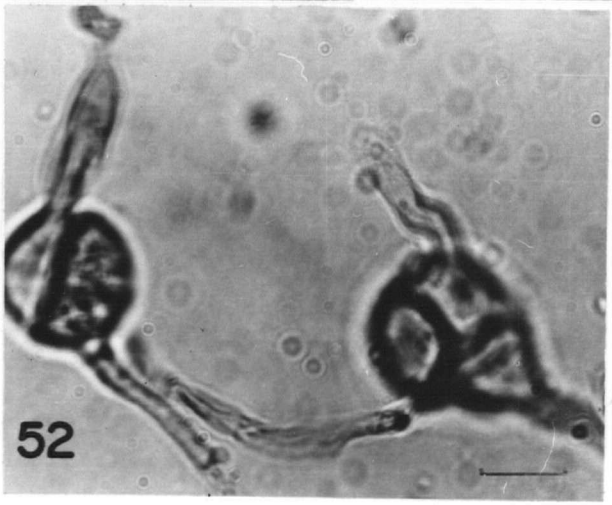
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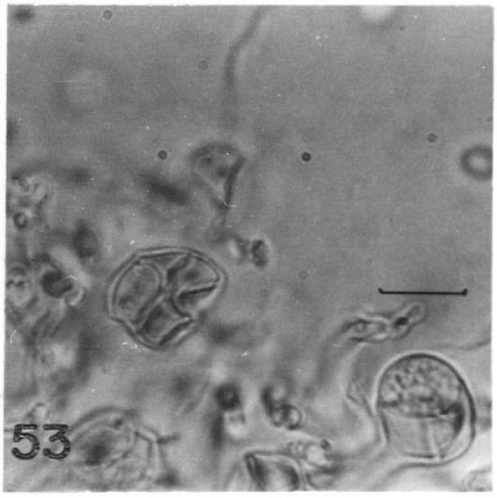
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Figures 54 - 58

Formation of epibasidia in culture.

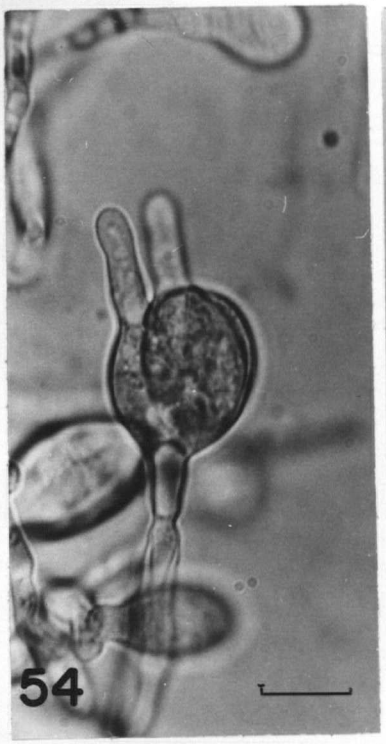
Fig. 54. Early stage in the formation of epibasidia.

Fig. 55. Two epibasidia, both irregular and nearly empty of protoplasm. Note septation.

Fig. 56. A basidium with a single epibasidium that has formed budding cells.

Fig. 57. Epibasidia showing variation in length. One epibasidium is atypical, with formation of two septa.

Fig. 58. A typical basidium showing epibasidia in different stages of development. One epibasidium is empty and collapsed, indicating possible basidiospore discharge.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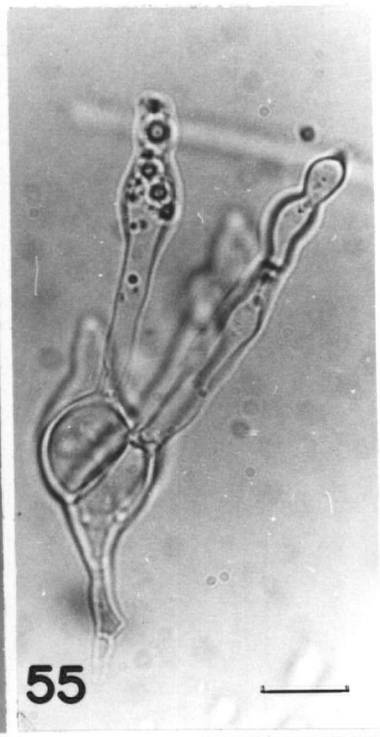
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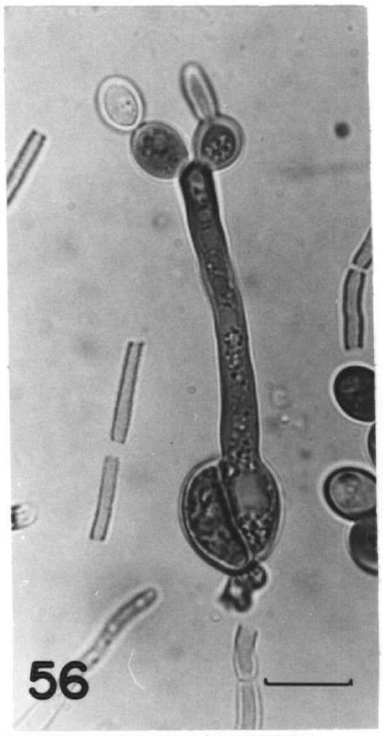
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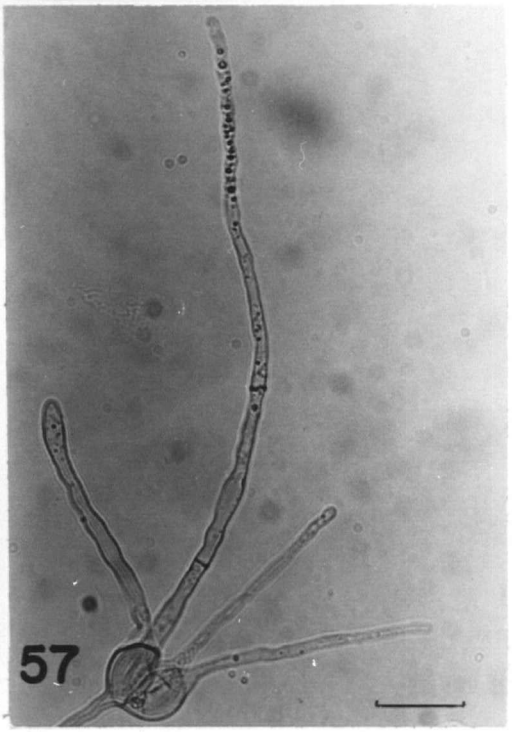
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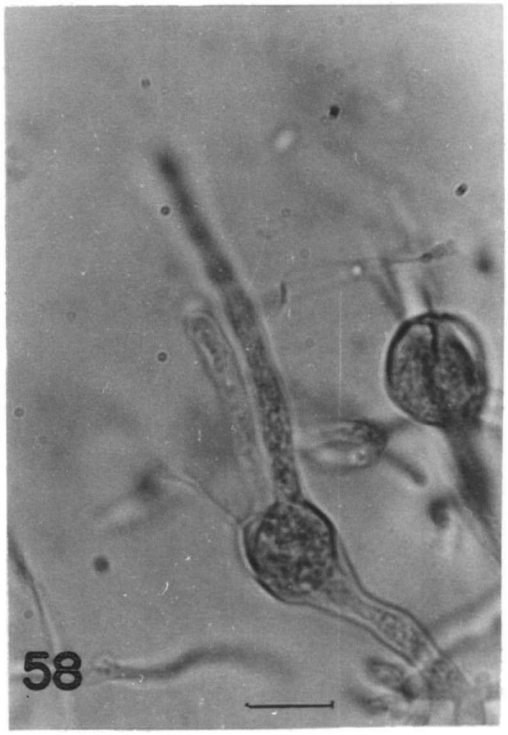
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basidia and degree of basidial development. Abundant basidial production was not random among strains but always occurred among specific ones; other strains consistently produced few basidia. Basidial initiation was frequently observed from some compatible strains within 10 days after crossing; in contrast, other strains produced no basidia during 6 months of observation.

Aborted probasidia subtended by a clamp, were borne terminally (Fig. 59) and laterally (Fig. 60) upon the dikaryotic hyphae. They frequently were abundant throughout the agar and basidiocarps in culture, as well as throughout basidiocarps in nature. These aborted basidia were conspicuous and, while septa or epibasidia were never completely formed, a single apical extension often developed (Fig. 59). Epibasidia and septa appeared to develop only from terminal probasidia.

Probasidium-like structures, not subtended by clamps, infrequently appeared upon dikaryotic hyphae (Fig. 61). These were of the general size and shape of basidiospores, but they were never observed to be discharged or detached.

Attempts to induce consistent and rapid development of basidia with the regular formation of septa and epibasidia were carried out using various nutritional and environmental conditions. Some of these experiments have been described previously in the results of experiments tabulated in Table XIX. In some instances, there were scattered pockets of developed typical basidia but this was not always consistent

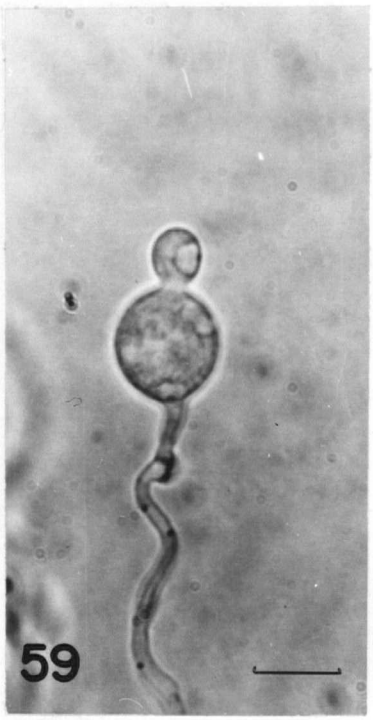
Figures 59 - 61

Formation of aborted basidia and basidium-like structures.

Fig. 59. Terminal probasidium with an extension developed from the tip.

Fig. 60 Lateral probasidia showing basal clamps.

Fig. 61. Probasidium-like structure. Note the absence of clamp and the strong resemblance in size and shape to a basidiospore.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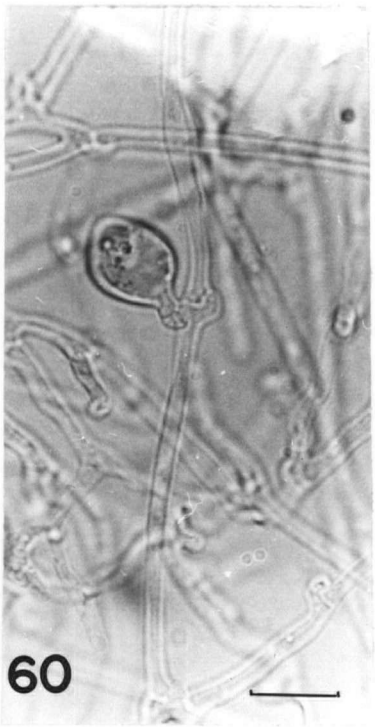
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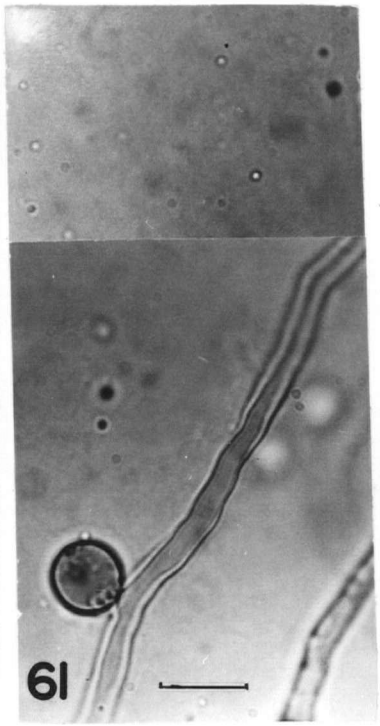
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among replicates. Dikaryotic stocks 543 X 2, 711 X 5, and 711 X 8 had been found previously to be consistent producers of large numbers of basidia and epibasidia. As indicated in Table XIX, vitamin fortified media resulted in more probasidia with septa, but the epibasidia often were branched, septate and non-functional. Normal epibasidia were most frequently observed where asparagine was added to the medium. No increased basidial development resulted where either PSA, water agar, or weak MYS medium was used as a substrate for the inoculation of basidiocarps, where basidiocarps established on MYS were dried for 3 days and revived with the addition of sterile distilled water, or where the position of the basidiocarp was varied. In the absence of light, basidia appeared very slowly in MYS medium and rarely developed epibasidia; if septa were produced, they appeared to be abnormal. Basidia developed more rapidly when cultures were incubated under light.

D. Basidiospore development

Large globose or sub-globose basidiospores were produced at the tips of the epibasidia in culture. They were formed as blebs, enlarged and became broadly apiculate near the point of attachment. Basidiospores seldom were found attached to the epibasidia in slides prepared from basidiocarps. Discharged basidiospores were most easily detected by observing the agar surface surrounding the basidiocarp or the inside surface of the lid of the inverted Petri plates. The first

macroscopic indications that spore discharge had taken place was the appearance of numerous, small, haploid colonies on these surfaces. The basidiospores germinated on the agar medium or in condensed moisture, could be detected by covering the particular surface with a coverslip and examining with a microscope.

In attempts to influence basidiospore production, replicate stocks from a single culture were inoculated on MYS and incubated at 28°, 15° and 3° C. Alternating periods of florescent light and dark in a 15° C. growth chamber or continuous incadescent light at 20° C., were used to incubate similar stocks on MYS or PDA. After a comparison of all plates under all conditions, no increased basidiospore production was evident. Similar negative results were obtained when sections of a single stock were grown on either MYS + *Valsa* extract, CJA, CJA + *Valsa* extract, PSA or Oat agar at 15° C., under alternating 12-hour light periods of florescent light.

The medium in Petri plates containing MYS and bearing numerous developing basidiocarps (stock 711 X 8) was cut into four quarters. Each section was transferred to plates containing either (1), MYS; (2), MYS with small holes in the top edge of the plate to provide for increased circulation of air; or (3), distilled water. Parafilm was used to seal plates containing MYS only. In 16 replicates of the four separate stocks, basidiocarps continued to develop but no

basidiospore production was obtained over the 20 day observation period.

Basidiocarps from stock 711 X 8 were grown on MYS for a period of 1 month. Basidia were found in various stages of development but basidiospores were not abundant. Eight cultures were dried by partially lifting one corner of the Petri plate lid, allowing the medium and basidiocarps to dehydrate. After drying 1 week at 15° C., either autoclaved distilled water was added to rehydrate cultures or, dried basidiocarps were transferred to fresh medium and incubated for an additional 15 days. At the end of that period there was no detectable basidiospore production. Microscopic observation indicated no further degree of maturation of basidia.

In summary, cultures transferred to new medium, incubated at 20° C. under a 60 watt incadescent lamp, produced the most abundant and typical basidiocarps, basidia and basidiospores. The minimum time observed, after transfer of stock to discharge of basidiospores, was 15 days; this took place in MYS medium.

DISCUSSION

I. Compatibility studies

The compatibility systems of fungi are described and interpreted in the recent general works of Hawker (1957), Esser and Raper (1965), Raper (1966a, 1966b), Esser and Kuenen (1967) and Burnett (1968). Little is known concerning the compatability systems of the Heterobasidiomycetes other than for the rusts and smuts.

Early compatibility studies on members of the Tremellales were carried out by Barnett (1937) on *Auricularia auricula* (Hook.) Underw. and several species of *Exidia*. He combined haploid phases of these fungi then later searched for the presence of clamps. While his results were not always clear, they indicated that the species examined possessed a bipolar type incompatibility system. The bipolar pattern was also reported for another Tremellaceous fungus, *Aporpium caryae* (Schw.) Teixeira and Rogers, by Macrae (1955).

Whitehouse (1949), from studies of 230 Hymenomycetes and Gasteromycetes reported, found that approximately one-third are heterothallic bipolar in nature, approximately one-half are heterothallic tetrapolar, while the remaining are homothallic. The distribution of known patterns as tabulated by Esser and Kuenen (1967) fits this estimate well. Bipolar and tetrapolar mating systems have been reported for the same species by different authors, but this conflict may be due to taxonomic obscurity, or to other problems such as the presence

of clamps only outside the area of the contact zone (Takemaru, 1961). In *Tremella*, the limited growth of the haploid hyphal phase, as well as the easily identified clamp connections, reduced the possibility for error in successful mating observations. Because of the submersed and concentrated growth of the dikaryotic mycelium, successful matings could be detected in most instances prior to microscopic observation. Seven to 10 days incubation is now considered adequate for determining consequences of pairing.

The tetrapolar mating type pattern that emerged from this study of *T. bambusina* resembles that of *T. mesenterica* (Bandoni, 1963) and of *Ustilago zeae* (Rowell, 1955). It is suggested that the control of conjugation tube formation in *T. bambusina* is the result of a single pair of alleles ("A" and "a") while fusion and dikaryon formation is controlled by multiple alleles or multiple factors ("B_1", "B_2", "B_3" and so on). In all other tetrapolar forms, compatibility is controlled by multiple factors designated as the A and B factors. Both A and B, in this system, involve two subloci each with multiple alleles. While the numbers of incompatibility factors appear rather low in some fungus groups (around 10 in the Gasteromycetes) the Hymenomycetes have as many as an estimated 350-450 factors for any one series (A or B) (Raper, 1966). No common "B" factors were detected among isolates from seven collections that I studied. The calculated number of "B" factors (14) for the seven collections, found within

a very narrow geographical area, would indicate that the "B" factors for *T. bambusina* could be numerous.

The distribution pattern that emerged from *T. bambusina* pairings indicate independent assortment for the "A" and "B" factors and thus these two factors are not linked. The expected distribution in a 1:1:1:1 ratio of the four mating types was always closely approached where sufficient spores were isolated from collections studies.

Three basidiocarps were collected on the same branch within a few centimeters of one another (collections SGB 620A, 620B, 620C). On another single stem, the maximum vertical distance between basidiocarps in collection 625A and 625B, was less than 15 cm. The interfertility studies indicated that each basidiocarp was the result of a separate and distinct mating, for none of these basidiocarps possessed the same "B" factors. Since single crossovers can result in new "B" factors, it is possible that a single mating can give rise to four "B" factors. However no crossovers were detected in the numerous isolates from any of the basidiocarps recorded here. The fact that basidiocarps may be close, or even confluent with each other, does not necessarily mean that they possess the same genotypes.

Compatibility in *T. bambusina* as in other fungi, could only be ascertained through the pairing of haploid strains. An exception to this was reported by Kendrick and Holton (1956) for the smut species *Tilletia caries* (DeCondolle)

Tulasne. Here, pigmentation of the medium has been used as an indicator of compatible types. Differences in consistency and color in some haploid strains of *Tremella bambusina* were not characteristic for any mating type but probably represent differences in moisture content and age of the colonies.

II. Basidiospore germination

The basidiospores of *T. bambusina* germinate readily in distilled water, as do basidiospores of rusts and smuts. Spores germinating in water undergo little or no swelling prior to the emergence of tubes (Hawker, 1966b).

No direct evidence of basidiospores themselves producing conjugation hormones has been proven but, with a similar nuclear condition, and similar methods of germination as budding cells, there is little reason to suspect that they do not. Basidiospores appear capable of direct conjugation between compatible pairs through conjugation tube formation.

The formation of a ballistospore on a sterigma-like outgrowth from a basidiospore has been reviewed by Donk (1956). Hawn and Vanterpool (1953) reported that basidiospores in *Rhizoctonia solani* Kuhn, underwent repetition in water but produced germ tubes on rich medium. Flentje (1956) reported repetition of basidiospores of the same organism on soil extract medium. Repetition in *Cronartium ribicola* Fischer was affected by pH (Bega, 1960). In *Rhizoctonia solani*, Whitney (1964) found that the mycelium produced a substance soluble in ether, causing repetition of basidiospores.

Factors present on the surface of the basidiocarp of *T. bambusina* may cause the majority of spores deposited there to reproduce by repetition. Germination by budding, similar to that seen in water or nutrient media, also is sometimes common on the basidiocarp and may be caused by the accumulation of moisture on the hymenial surface, as suggested by Kobayasi and Tubaki (1965).

The one observation made of a basidiospore germinating by repetition in moisture accumulated on the inside lid surface of an inverted Petri plate can be explained by the carrying of a previously discharged and germinated spore from the basidiocarp surface by the abstriction of other spores. It seems conceivable that the formation of basidiospores from haploid metabasidia result from substances and/or conditions of the hymenial surface. These factors seem to influence discharged haploid basidiospores resting on the hymenium, to produce ballistospores in a manner similar to metabasidia. Whelden (1934) states that, although difficult to detect, tertiary spores are apparently also formed. An interesting experiment, once a reliable source of basidiospores can be obtained in culture, would involve the reaction of spores and budding cells on a permeable membrane covering basidiocarp surfaces.

III. <u>The haploid phase</u>

Requirements for the growth of the haploid phase of *T. bambusina* do not differ greatly from the general nutritional

patterns and physical conditions reported for the growth of other basidiomycetes. It has been clearly demonstrated that fungus nutrition can be affected by the elements in solution and O_2/CO_2 ratios as well as various physical factors including pH, temperature and light. It is common knowledge that the nutritional conditions for the growth of a fungus often vary from species to species, from collection to collection and from strain to strain. Cochrane (1958) and Lopez and Fergus (1965) reported different isolates of the same species differed in their abilities to utilize the same compound. Initially I thought that this might be the case with respect to differences of growth between media with or without added calcium. The use of synthetic medium lacking a calcium compound led to the investigation of the role that calcium played in the growth of *T. bambusina* and the subsequent preliminary assay of other major ingredients of the medium. The experiments on synthetic media were carried out exclusively with the haploid phase because of the inability to maintain the dikaryotic phase in liquid culture. The mycelial phase will grow on artificial media but it grows slowly and reverts quickly to the faster growing budding phase when placed in a liquid medium. It was not the intent of these experiments to determine whether or not the requirement for calcium was absolute, a much closer study must be made to perceive this. The results that were recorded, give some clues for future investigations of this problem.

A. The role of calcium

A partial list of some of the more recent investigations on the effects of calcium on fungal growth is presented in Table XX. Few basidiomycetes have been investigated in this respect. The role of calcium in the growth of fungi has not been resolved and the results of different investigators are often conflicting. Some of the conflict might be caused by contamination, the use of different media, or differing strains of the same fungus.

In early experiments with *T. bambusina* there may have been some calcium ion contamination in the control medium; however, this medium resulted in little growth compared to media with added calcium compounds. The results appeared significant and attempts to refine the medium and reduce possible calcium contamination were planned.

The most obvious source of contamination by calcium ions were the traces of impurities found in inorganic salts used in preparing media. The four-element microelement solution of SMD, substituted for the more complex Vogel's trace element solution used in previous media, helped to reduce the possible sources and amount of calcium contamination.

Other sources of calcium ions, not so obvious, might be the glucose and asparagine. Fries (1956) analysed an autoclaved glucose-asparagine medium and found various metallic elements, including calcium, precipitating out at different pH levels. The use of citric, tartaric and lactic acids as chelating agents for the binding of this element was suggest-

Table XX. Recent reports of the role of calcium compounds in fungus growth.

Calcium did not stimulate growth substantially

Aspergillus niger Van Tiegh, (Steinberg, 1948)

Fusarium oxysporum Schlect var. *nicotinae* Johnson,(Steinberg, 1948)

Chaetomium globosum Kunze, (Basu, 1951)

C. brasiliensis Batista & Pontual (Basu, 1952)

Phytophthora cinnamomi Rands, (Chee & Newhook, 1965)

Rizoctonia solani Kuhn, (Erwin, 1968)

Calcium stimulated growth

Rhizoctonia solani Kuhn, (Steinberg, 1948)

Sclerotium rolfsii Sacc., (Steinberg, 1948)

Pythium irregulare Buis, (Steinberg, 1948)

Allomyces sp., (Ingraham & Emerson, 1954)

Phytophthora spp., (Lopatecki & Newton, 1956)

Coprinus spp., (Fries, 1956)

Phytophthora sp., (Erwin & Katznelson, 1961)

Morchella crassipes Vent ex. Fries, (Robbins & Hervey, 1965)

Phytophthora cinnamomi Rands, (Erwin, 1968)

ed. A citrate buffer was added to the calcium-deficient medium in one experiment to reduce the availability of calcium ions as well as to maintain a more constant pH level. Results were not noticeably improved and the use of citrate buffers was terminated. Inconsistent results led to further attempts to purify the medium. Rochford and Mandle (1953) reported the removal of magnesium and calcium ions by passing medium through an Amberlite 1R 120 resin column. A sucrose + asparagine solution was passed through such a column by Erwin (1968). He used (1) the filtrate of this solution or (2) the solution prior to filtering, to prepare two calcium-deficient media. He found there was a significant decrease in dry weight of what appeared to be a calcium requiring fungus *Phytophthora cinnamomi*, when grown in medium containing the filtered sucrose + asparagine solution. The substitution of ammonium tartrate in SMC and SMD in this study was an additional attempt to eliminate possible calcium contamination from asparagine.

It appears that while calcium might not be a basic nutritional requirement, it may serve other roles in the growth of some fungi. In their experiments with *Aspergillus oryzae*, Simonart and Chow (1954) suggested that an effective buffer is released by calcium salts, aiding in the formation of basic amino acids and in decarboxylation of glutamic acid with the production of alpha-aminobutyric acid. Lindeberg (1944), working with species of *Marasmius*, reported that

calcium protects against injurious effects of some negative ions such as sodium, potassium and hydrogen. Calcium influenced the growth of *Aspergillus oryzae* when grown in a medium of high sodium concentration (Simonart and Chow, 1954) but had no effect upon the growth of *Aspergillus niger* in medium of low sodium content, (Steinberg, 1948).

Besides affecting the assimilative growth, calcium has been shown to influence conidial formation in *Trichoderma viride* and *Penicillium notatum* (Brian and Hemming, 1950; Hadley and Harrold, 1958) and the formation of perithecia in two species of *Chaetomium* (Basu, 1951, 1952).

In early experiments with *T. bambusina* where equivalent amounts of strontium were substituted for calcium, similar increases in growth were recorded. Erwin (1968) reported that strontium completely replaced calcium in the nutrition of *Phytophthora cinnamoni*; Ingraham and Emerson (1954) found a similar situation when strontium was substituted for calcium in the nutrition of a strain of *Allomyces arbuscula* Butler.

The rapid growth of the haploid cells took place in calcium deficient media when the technique of preparing the inoculum at a reduced temperature and precooling the medium prior to inoculation was carried out. This seems to indicate that calcium is not required by *T. bambusina*. I can, at present, only speculate concerning the reasons for the early observations of the lack of growth in calcium deficient

medium. Evidence appears to point toward temperature conditions, but such investigations were beyond the objectives of this thesis.

Calcium could conceivably protect the living cells against high temperatures or could provide for the availability of some component of the medium at the higher temperatures. Many of the early experiments in this study were carried out at room temperature in the summer months when the laboratory temperature often exceeded 30° C.

Damage to the cells might have occurred during the preparation of the inoculum. The solution being prepared was shaken by hand for a period, at times, of 10 to 15 minutes or more before the cells could be fully dispersed and the temperature of the inoculum may have approached 35° C. This temperature is considered to be near that which is lethal for *T. bambusina*. The cells remained in the inoculum suspension for varying degrees of time depending upon the quantity of medium to be inoculated and the order in which the medium was inoculated.

B. Nitrogen requirements

The haploid stage of *T. bambusina* grew well with an ammonium compound as the sole source of nitrogen. Nitrogen in the form of a nitrate appeared to be a less useable source and this observation is common for basidiomycetes that have been studied (Cochrane, 1958). Lilly (1965) felt that most fungi could use an inorganic nitrogen source and Nicholas

(1965), in a review of the utilization of inorganic nitrogen compounds by fungi, felt that most groups could utilize nitrate, but this ability was often induced by various culture conditions. Apparently the nutritional aspects of SMD and/or the physical conditions were not provided for nitrate utilization by *T. bambusina*. This species appeared much less exacting in respect to nitrogen requirements than the three species of *Tremella* studied by Flegel (1968). He reported that *T. encephala*, *T. subanomala* and *T. mesenterica* grew poorly on media with ammonium nitrate as a nitrogen source. The results of nitrogen utilization in *T. bambusina* is the same as that reported for *T. fuciformis* (Kobayasi and Tubaki, 1965) where ammonium sulphate was assimilated but potassium nitrate was not.

When fungi are grown in solutions that contain ammonium salts of strong inorganic acids, eg. ammonium sulfate, as used in medium SMB, this medium becomes more strongly acidic during growth. The uptake of the ammonium ions by the cells (Barnett, 1937) accounts for most of the early drop in pH but this can be minimized by using an ammonium salt of a weak acid. The subsequent use of either the alpha amino acid + l-asparagine or ammonium tartrate as the nitrogen source in SMC and SMD was adopted and resulted in a higher pH value for a longer period of time while still providing for rapid growth.

C. Carbon requirements

In SMC, d-glucose, sucrose and maltose appeared to be effectively utilized while the carbon of sodium acetate did not appear to be used. Kobayasi and Tubaki (1965) tested a greater number of carbon compounds and found *T. fuciformis* assimilated glucose, sucrose, maltose, sodium acetate, glactose, ethanol and cellobiose. Lactose or soluble starch were not utilized. The growth produced by *T. bambusina* in starch solutions could have resulted from the breakdown of this compound during autoclaving. Because the objective of the experiments was to determine which carbon source is effective in the growth of *T. bambusina* and not whether a carbon source can be utilized, the sugars were added to the medium prior to autoclaving. The breakdown or carmelization of some sugars during the autoclaving process is universally recognized and could inhibit growth. Negative results, if present, of sugar assimilation experiments could be invalid.

D. Vitamin requirements

Fries (1965) states that a single strain is usually considered representative of the species as far as growth factors (vitamins) are concerned. The vitamin thiamine is needed for good growth of the haploid phase of *T. bambusina* as has been shown in many basidiomycetes. Until serial subculture have been carried out on deficient media, little can be stated as to the need by this fungus for other vitamins. Good growth was continually evident however when medium con-

taining the two vitamin addition of thiamine and biotin was employed. The weak growth in the control medium indicate little carry-over of vitamins from the inoculum. As pointed out by Lilly and Barnett (1951), temperature, pH and composition of the medium are known to influence the effects of vitamins upon growth of various fungi.

E. pH requirements

No attempt was made to determine absolute pH requirements of *T. bambusina*. However, a study was made to determine at which initial pH level rapid haploid cell growth would occur, and the changes that took place in the medium and the cells. It is recognized that such factors as temperature, types and components of media, and O_2/CO_2 ratio and conditions of pregrowth can alter the pattern indicated and that the results of these experiments do not necessarily indicate similar patterns for the dikaryotic phase.

No attempt was made to regulate the pH of the medium by buffers. I felt that the pH change that occurred over a period of 3 days was not important in this study. In these experiments, because the initial amounts of inoculum were small and quantity of medium was increased, the drop in pH was reduced. Fries (1956) has shown that a restriction of fungal growth may occur with the use of excessive concentrations of buffer, and the chemical composition of the medium often is altered.

Results of the pH studies agree reasonably well with

those made by Flegel (1968) on three other species of *Tremella*. There was no pH change in liquid CJL from the initial levels of pH 5.5 and 7.0. In MYS there was no change at pH 3.0. Initial pH values below these levels tended to raise while pH values immediately above these figures tended to be lowered during the 3 day growth period. These pH levels are similar to those providing the bimodal growth pattern observed by Flegel using a range of buffered pH solutions. Fries (1965) reported that a similar bimodal pattern, in the cultivation of *Coprinus*, resulted from the precipitation of specific ions at different pH levels. The rapid drop in *T. bambusina* growth rate, in the basic pH range, is dramatic and similar to that reported by Flegel.

T. bambusina grew well between pH 3.0 - 7.0, a range slightly broader than that characteristic of most fungi (Lilly, 1956b). The lack of growth of *T. bambusina* at pH 2.0 does not correlate well with Flegel's findings of substantial growth at pH 2.1 with three other species of *Tremella*. Medium with an initial pH of 8.0 or 9.0 produced good growth because of an early drop in pH to a more suitable level. Optimal growth near pH 7.0 is typical of a few fungi (Cochrane, 1958) but not of the species investigated by Flegel. His graphs indicate a pH level of near 4.7 to be optimal. These differences could be attributed to species differences, or differences in media, temperature, or other factors.

Kobayasi and Tubaki (1965) reported, from growth experi-

ments with *T. fuciformis* at temperature levels of 10°, 25°, and 37° C., maximum growth at 25° C. This is near the optimum range found for the dikaryotic phase of *T. bambusina*.

IV. Conjugation phase

A. Formation of conjugation tubes

The conversion of budding growth to conjugation tubes in *Tremella* has similarities to the dimorphic condition of hyphal and budding growth common among the fungi. Much of the recent work on dimorphism in the Phycomycete genus *Mucor* has been reported by Bartnicki-Garcia and Nickerson, (see Bartnicki-Garcia, 1963 for a review and partial bibliography). The genus *Candida* in the Fungi Imperfecti (Cryptococcaceae) is the most intensively studied of all the dimorphic fungi. In the Ascomycetes some of the true yeasts in the Endomycetales are characterized by the formation of pseudomycelium under certain conditions and those normally hyphal in nature often become yeast-like when grown in concentrated sugar solutions. Yeast to mycelium conversion is also common in species of the Taphrinales. Similar dimorphic conditions are common for the Ustilaginales as well as the following genera of the Tremellales; *Sirobasidium, Tremella, Naematelia, Tjibodasia, Myxogloea, Xenolachne* and *Septobasidium* (Kobayasi and Tubaki, 1965).

Control of morphogenesis may be exogenous and has been experimentally manipulated by changing factors in the environment. Dimorphism is known to be influenced in specific

fungi by conditions of temperature, nutrients, age, aeration, and some chemical compounds; e.g. camphor, antibiotics, cobalt, boron and carcinogenic chemicals (Scherr and Weaver, 1953; Cochrane, 1958; Ramano, 1966).

Early investigations on species of *Saccharomyces* and *Blastomyces dermatitidis*, indicated that the lowering of temperature below the optimum for yeast growth, resulted in the conversion of yeast to mycelium. This has been frequently substantiated and termed "thermal dimorphism" (Nickerson and Edwards, 1949). There is no report of change in form when *T. fuciformis* haploid yeast phase was grown at 10° and 25° C. (Kobayasi and Tubaki, 1965). No conversion to mycelial phase has been noted in *T. bambusina* grown at various temperatures, but further study needs to be made to substantiate this and to detect if there is a change in cell morphology.

The reducing sugar, glucose, has been shown in *Candida albicans* (Nickerson, 1951; Wickerham and Rettger, 1939) to influence the conversion of mycelial phase to the yeast phase. Because *T. bambusina* utilizes non-reducing sugars, the incorporation of sucrose in conjugation medium for growth of the mycelial phase, may be a better choice. Kobayasi and Tubaki (1965), used a sucrose carbon source for growing *T. fuciformis* basidiocarps.

Although no effects have been noted, careful consideration of the nitrogen source needs to be made, for it may

also enhance the development of *T. bambusina* yeast or mycelial phase. Cysteine induced conversion of mycelial to yeast types in *Candida albicans* (Nickerson and Van Rij, 1949), and cysteine and adenine, when added to medium containing aspartic acid, resulted in long cells being produced by *Ustilago sphaerogena* (Wachter and Spoerl, 1961). Spoerl, et al. (1957) studied the effects of amino acids upon the length of cells in *U. sphaerogena*, and found that glycine, serine and threonine produced long mycelial-type cells. This species normally exists as short rods in media containing other amino acids.

Aging of cultures has been known for a long time to effect morphology differently in different media. In many instances this conversion in form has been attributed to the O_2/CO_2 concentrations, food supply or other factors, but can also be due to the lowering of pH to below an optimum for yeast growth. This was demonstrated in *Saccharomyces* (Scherr, 1953) and had been reported earlier for other yeasts. pH influences the growth of *T. mesenterica* conjugation tubes (Flegel, 1968). While he showed growth of the haploid phase at pH 2.1 and 7.2, there was no initiation of conjugation tubes at these same levels. Flegel concluded that the pH necessary to bring about optimal growth of conjugation tubes also was that level resulting in optimal growth of the haploid phase. The ability of *T. mesenterica* to conjugate easily in liquid media does not appear to be characteristic

of *T. bambusina*. Under the conditions used, the number of conjugation tubes initiated never approached that of *T. mesenterica* as reported by Flegel. He observed that over 50% of the cells produced conjugation tubes at pH 4.7 and that no tubes were initiated at pH 7.2. pH could account for the failure of *T. bambusina* to form tubes in liquid CJA and MYS, with an initial pH near the 7.2 level. However cells from liquid CJA readily formed tubes when placed on solid CJA with a pH of 7.2. This apparent anomaly could be caused by impurities in agar, aeration or the stationary conditions of the cells.

Flegel also reported that the length of time a strain has grown since last transferred to agar medium, as well as the age of the solid medium, were important factors which could affect the degree of conjugation tube production. The age of the culture or the age of the medium does not appear especially critical for *T. bambusina*. No detectible differences in tube numbers or growth appeared when 2 or 30 day-old cultures were used or where 1 or 30 day-old agar medium was employed. Differences in degree of inoculum consistency may help account for the degree of conjugation tube formation. Inocula from young cultures, being soft, could be more easily spread across the surface of the media and would ensure a greater degree of mixing with the opposite mating type. The extreme dehydration of older media, of course, is noticeable, especially where a compatible pair had been previously shown to be a weak producer of conjugation tubes. Likewise

the older strains of such pairs could result in a greater chance of observed lack of conjugation.

In *T. bambusina*, the inhibition of conjugation tube and hyphae, by the haploid phase appears similar to that reported by Lingappa et al. (1969) where substances produced by the yeast phase of the dimorphic fungus *Candida albicans* inhibited its mycelial growth. Phenethyl alcohol and tryptophol were found to be the active inhibitors of *C. albicans* mycelial growth. Terenzi and Storck (1969) also found phenethyl alcohol to be an effective inhibitor of mycelial growth in five species of *Mucor*.

Scherr and Weaver (1953) have discussed several concepts postulating mechanisims for dimorphism. The concept that the process of cell division and cell elongation involve independent mechanisms was postulated to account for filamentation in bacteria (Hinshelwood, 1946). It is now generally concluded that the process of cell division is separate and distinct from synthesis of new protoplasm, and that sulfhydryl compounds affect the process of cell division. This appears true for numerous animal and plant cells investigated, and probably plays a major role in dimorphism of fungi too (Romano, 1966). Much support has been given to the report of Nickerson and Van Rij (1949) who indicated that when reduced organic sulfhydryl compounds are available to yeasts, rapid cell division is favored and budding results. If the sulfhydryl compounds in the cell are not reduced, cell divi-

sion would be inhibited, resulting in filamentous growth. Nickerson and Falcone (1956) have postulated a specific cell division enzyme in *Candida albicans* resulting in the reduction of disulfide covalent bonds in the glucomannan-protein complex of the walls and a subsequent conversion to a budding phase.

B. Conjugation hormones

Little work has been reported to account for the interconversion of budding and hyphal formation by hormone systems. Most investigations have emphasized the temperature, aeration and nutritional aspects of dimorphism. Hormones controlling the change from the haploid phase to a hyphal phase, in the form of conjugation tubes, was hypothesized by Bauch (1925) for *Ustilago bromivora* (Tul.) F. de W. After observing the development of conjugation tubes between plus and minus strains of *Saccharomyces cerevisiae* Meyen ex Hansen in liquid culture, Levi (1956) published experimental evidence that conjugation tubes were hormonally directed. Minus cells were induced to form conjugation tubes when placed near plus cells or on agar from which active plus cells had been grown and removed. Minus cells could not evoke recriprocal response from plus cells. Yanagishima (1969) has reported the isolation of hormone-like substances from the culture media of haploid *S. cerevisiae*. These substances, produced by both mating types causes activation of cell-wall softening and the expansion of cells of the opposite mating type. This reaction

appears preliminary to conjugation and is genetically controlled.

In the species of *Tremella* examined, Bandoni (1965) first reported that the shifting from budding to hyphal growth results from substances released by the opposite mating type. This was confirmed by Flegel (1968) and in this thesis, but the exact nature of these substances and their mode of action awaits further study and clarification. The ability of *Tremella* to produce conjugation hormones continuously without the need of stimulation by the opposite mating type, and the theory that this hormone is used or altered during tube growth, appears to hold for all *Tremellas* thus far studied. Flegel (1968) reported, that in *T. mesenterica* the reversion of conjugation tube growth to budding, resulted when the conjugation hormone was removed. From my observations, it appears that the same responses are brought about by heat and light, possibly through the alteration or destruction of the hormone by these factors. Why some daughter cells form tubes and other adjacent daughter cells from the same parent do not is an interesting phenomenon and may be due to the degree of maturation of each cell.

High temperature affects hormone structure. The report of the stability of the tube initiation hormone(s) in *T. mesenterica* to autoclaving reported by Bandoni (1965) could not be duplicated with any strain of *T. bambusina* used, at any pH or temperature level tested. The inhibitory effect of a dense concentration of the yeast phase upon the formation of

conjugation tubes account for some failure. Attempts to control this factor by using very small amounts of inoculum did not have any noticeable effect.

C. Fusion of conjugation tubes

The nature of the substances regulating directional growth of the conjugation tubes prior to their end-to-end meeting might be due, as suggested by Bandoni (1965), to diffusible agents secreted at the growing tips. If the effective area of growth is considered to be limited to the hemispherical tip of the conjugation tube, then regulatory substances would be effective in this same area. A positive tropic response between conjugation tubes would result from an increase in the extensibility on the side of the apex nearest the source of the diffusible substance (Robertson, 1965b). The response occurs in tubes of both mating strains and the substance(s) appears to be labile. There is no response evoked in an area shortly after the conjugation tube has grown beyond that point.

The effective distance of the diffusible substance(s) controlling the end-to-end meeting of hyphal strands prior to conjugation, is similar to that measured in various species of Basidiomycetes studied by Buller (1933), Gregory (1966) and Bandoni (1965). In *Tremella*, unlike most other fungi studied, the fusion takes place between conjugation tubes rather than between infrequently occurring lateral branches of mycelia.

I have noted that the increased production of conjugation tubes does not necessarily result in a comparable increase in conjugation and formation of the dikaryotic phase. The pH or other conditions optimal for the growth of conjugation tubes may not be those optimal for the effectiveness of directional growth substances and the control of fusion or subsequent growth of the dikaryotic phase. I have not observed tropic responses at the tips of conjugation tubes of paired mating types compatible for tube formation only.

Fusion can occur between compatible cells in close proximity to one another without the formation of visible conjugation tubes by one or both pairs. Triple fusions were not observed, but theoretically could occur. Because of the limited growth of the conjugation tubes, there does not appear to be the problem of migration of nuclei through the preformed hyphae as occurs in many Basidiomycetes.

V. <u>The dikaryotic phase</u>

The genetic factors controlling conjugation regulate the establishment of the dikaryotic condition in *T. bambusina*. Once the dikaryotic phase has been established other genetic and environmental factors influence growth of this phase and its transformation into various structures. Most research with other fungi involves physical factors such as moisture, pH, light, temperature and CO_2 concentration, nutritional requirements including mineral and vitamin, and genetic factors.

The dikaryotic hyphae characteristically grow through the agar as well as upon or near the surface, helping to distinguish it from the monokaryotic hyphae. I expected O_2/CO_2 concentration to be a contributing factor to the behavior of these phases; however, no growth difference was noted when the colony was covered with a coverslip.

A. Dedikaryotization

The isolated dikaryotic phase in laboratory culture invariably became contaminated by the haploid budding phase. This presented a major obstacle to investigations of the dikaryotic phase, for the budding cells grew rapidly over the mycelium or became the major phase when transferred to new plates. These budding cells characteristically are formed as blastospores at the ends of the dikaryotic hyphae and are identical in appearance to those arising through basidiospore germination, from conjugation tubes, or from sterigma. When these conidia were paired with known mating types, the formation of conjugation tubes and conjugation was observed; this does not preclude the possibility of binucleate conidia being present. Neuhoff (1924) observed binucleate conidia from *T. folicea* and *T. lutescens*. Binucleate conidia, subtended by clamps, appear as arthrospores and have been described by Whelden (1934) and Bandoni (1958). Raper (1966a) briefly describes dedikaryotization of a number of basidiomycetes, but the exact process by which this is brought about in *Tremella* is as yet unsolved.

B. Basidiocarp formation

Early successes in developmental studies of the basidiocarp were achieved with agarics and polypores. Taber (1966) states that more than 150 species of agarics, polypores and boletes have been grown in laboratory culture and these forms, with few exceptions (*Hericium*, Yurchenco and Warren, 1961; *Cyathus*, Brodie, 1948, Olchowecki and Brodie, 1968; and *Sphaerobolus*, Alasoadura, 1963) remain the most intensively studied. Compared with the number of Basidiomycetes forming carpophores in nature, the number doing so in laboratory culture is small. Because of a lack of definitive structures such as stipe, pileus, gills, etc. found in other basidiocarps, a difficulty exists in ascertaining what is a normal basidiocarp in *Tremella*. Texture, consistency and microstructure appear to be important criteria here.

Growth of *T. bambusina* basidiocarps *in vitro* takes place under a highly artificial environment. Unlike the natural environment, a continuous supply of water is available, and the composition of the atmosphere and relative humidity are highly modified. The accumulation of staling products in the substratum and air can affect precise stimuli required at different morphogenetic phases during development.

Basidiocarp production appears to be controlled by genes other than mating factors, since certain compatible strain combinations always produced few or no basidiocarps. Other dikaryotic stocks regularly produced basidiocarps under

identical conditions. The capacity to form basidiocarps in *Collybia velutipes* (Curt.) Fr. was reported to differ with respect to various monokaryotic strain combinations (Aschan, 1954; Aschan-Aberg, 1960a, 1960b). Others (Gilmore, 1926; Eugenio and Anderson, 1968) have also shown a genetic influence upon the capacity of specific fungi to form fruit bodies. In *Schizophyllum*, common A, common B and common AB dikaryons can be produced in culture, and all have formed basidiocarps.

Nutritional requirements for fruiting of species are varied and complex, and nutrients required by one fungus are not satisfactory for another. It has been shown that the quality and the quantity of nutrients, and their ratios, as well as the effects of other factors, (e.g. pH, staling products) on these nutrients play important roles. Generally speaking, most fungi studied obey Klebs' (1900) principle that reproduction, including carpophore formation, takes place under more restricted environmental conditions than vegetative growth. Restriction, i.e. decrease in the available food supply, is considered to be a common factor in inducing the formation of carpophores. This restriction can be brought about in ways other than the depletion of food by growth of the fungus. The use of complex carbon sources in the medium can prevent rapid hydrolysis (Hawker and Chaudhri, 1946) or phosphorylation (Hawker, 1947) by the fungus. Dilution of nutrient solution in large volumes of media was reported by Plunkett (1953) to be important in inducing fruiting of

Collybia velutipes.

Nutrients available in PSA, MYS or PDA, are adequate for basidial production, and light conditions or medium consistency does not appear to influence the quantity of basidiocarps. Humidity might eventually be shown to be important in the development of normal basidiocarps, but it is difficult to control this factor with the equipment at hand. Some media, where the agar was increased to 25 g./liter, had less available water and less condensate. This dry condition also developed in other media with increasing age of the plated medium, dependent upon the temperature of incubation and the closeness of fit of Petri plate halves. As the medium dried out, it is conceivable that an optimum point was reached, initiating a specific stage in basidiocarp formation.

There are about 20 species of *Tremella* reported to be parasitic or at least associated with other fungi (pers. comm., R. J. Bandoni). The relationship between *T. bambusina* and the associated pyrenomycetes occurs on species of at least two genera, *Valsa* and *Diaporthe*. This occurrence helps to confirm suspicions of many, that these two genera are more closely related than is indicated in the present classification. Madelin (1968) in reviewing the relationships of fungi growing on other fungi, found few reports of Basidiomycetes parasitic upon other fungi and stated that the hosts reported are gill fungi or Boletes. Exactly what the relationship is that exists between *T. bambusina* and *Valsa* or *Diaporthe* is

uncertain. The association must be classified as more than incidental, otherwise other locations in nature should support growth of this species.

It is difficult to identify with certainty, the species of accompanying ascomycete. The perithecial cavity of *Valsa* or *Diaporthe* is filled with the dikaryotic hyphae of *T. bambusina* resulting in the absence of asci and ascospores. Consequently, surrounding perithecia must be examined and these might not be the same species. The spores within a single perithecium of either genus of Ascomycete are variable and possess few distinguishing characteristics. Many species of *Valsa* appear in the literature and it is unknown how many are based on differences in the host on which they were described. Identifications are based, in part, on hosts. Three species were identified in Bandoni's collection labels, RJB 130, *V. ceratophora*; RJB 1561, RJB 1560 , *V. clavigera* Dearn & Barth.; and RJB 614, *V. abietis* Fr.

A general review of the effects of light and other factors on basidiocarp formation in the Basidiomycetes, is presented by Taber (1966). He has reported that while light requirements varied in respect to the initiation of basidiocarps, in most instances light is required for proper development of stipe or pileus and therefore normal spore production.

Light has not been definitly shown to play an important part in the initiation or development of basidiocarps of *T.*

bambusina. Bulat (1954) reported the influence of light upon the pigment formation of *Dacrymyces ellisii* in culture. A similar influence upon color does not exist in *T. bambusina*, for colored and colorless basidiocarps commonly appeared within the same culture from the same stock.

The pH range for basidiocarp production in the Basidiomycetes is more restrictive than that for vegetative growth in all species reported. This aspect of regulation in *T. bambusina* has not been studied as yet.

Aeration is reported to be important in basidiocarp production, especially because of effects on humidity and the level of concentration of the biproducts of metabolism. Studies on three different Basidiomycetes by Stoller (1952), Plunkett (1956), Long (1962) and Niederpreum (1963) have shown that a low CO_2 level is critical for the development of basidiocarps. No research has been carried out with *Tremella* with respect to O_2/CO_2 involvement with fructification but this factor could be involved.

C. Basidium formation

The basidium is considered to be a relatively constant structure and is given great importance in the classification of the Basidiomycetes, especially in the Tremellales. No critical studies concerning the effects of factors upon the development of basidia could be found in the literature.

Abundant production of spores is an indication of normal basidium development and the basidia, except for epi-

basidia length and septal position, are relatively constant in *T. bambusina*. If spores are not produced *in vitro* within 30 days after inoculation, inadequate culture conditions are present and abnormal basidia often develop. Septa appear to be irregular, epibasidial growth slows, stops, or branches, or narrower extensions form at the tip, or conidia are produced. These reactions are similar to those reported for *Fusarium*, where the hyphal apices were flooded with water (Robertson; 1958, 1959, 1965a) and where small droplets of water were placed on hyphal tips (Thimann and Gruen, 1960). In *T. bambusina*, when the haploid hyphal tips of the epibasidia reach the surface of the basidiocarp and are suddenly exposed, they generally form basidiospores. If the hymenial surface is wet, however, development at the hyphal tip could be quite different and merits further investigation. A similar reaction could also be caused by the constant basidiocarp size increase in culture, a factor not present in nature.

D. Basidiospore formation

Hawker (1966a) has discussed the physiological conditions affecting basidiospore production. Morton (1961) stated that vegetative growth is encouraged by high concentrations of nutrients and, even though the formation of basidia and basidiospores also require exceptionally large amounts, the source is from food material already accumulated by the fungus and not that which is awaiting absorption. There are clear indications that materials in the vegetative hyphae of

agarics are transferred to the developing basidiocarp (Madelin, 1956a, 1956b, 1960; Plunkett, 1961). Corner (1934) and Wessels (1966) suggested that cell wall constituents might also be involved in supplying material. Once the basidiocarp has been produced and spores initiated, the addition of nutrients might play only a secondary role. There is accumulating evidence that each stage in the development of a Basidiomycete can be regarded as providing a "substrate" and conditions for the development of the following stage (Hawker, 1966a), (Wessels, 1965). There have been few studies of nutritional effects upon sporulation of the more complex Basidiomycetes and these only through their influence on earlier developmental stages. Most experiments relating sporulation to nutrition have been carried out upon molds, where the effects can be more direct. *Tremella* basidiocarps, being likewise simple, could also be more directly influenced by outside nutrient sources. Once epibasidia have been established and have grown to the surface of the basidiocarp, factors which affect the translocation of nutrients to the spore and the growth of the hyphal tip into the basidiospore appear to be critical. Humidity and, perhaps, light seem to play a major role.

Although basidiocarps of *Tremella* are formed on a damp substratum, they can obviously survive considerable periods of desiccation and will commence to grow and sporulate again with a return to moist conditions. Drying is probably not a

prerequisite for basidiospore formation in *T. bambusina*. Where the agar and basidiocarp were allowed to dry, then transferred to fresh medium or rehydrated after 1 week, growth was rapidly reestablished but there was no observable difference in number and maturity of basidia nor was there increased formation of basidiospores. In all instances where abundant basidiospores were produced, dryer conditions existed as indicated by a minimal amount of condensation observed on the walls of the growth chambers.

The effects of aeration upon sporulation of fungi are reviewed by Lilly and Barnett (1951) and Hawker (1957, 1966a). The effects are attributed to the increase in O_2 concentration as well as the decrease in CO_2 or ammonia concentration *in vitro*. The requirement for increased aeration generally appears to be higher during reproduction than during vegetative growth for most other fungi. Sporulation of *T. bambusina* might increase with aeration of cultures, for abundant basidiospores formed after growth chambers had been uncovered for observation.

The effects of light upon spore initiation and development are reviewed by Lilly and Barnett (1951), Hawker (1966a), Taber (1966), Page (1965) and Pomper (1965). There are some species where light apparently has no affect upon basidiospore production while other species have an absolute requirement for light. Light does not appear to be necessary for spore discharge or any stage after the growth of the epibasidia in

T. bambusina. However, transfers and incubation of cultures was not carried out in a dark room.

Mature sporulating basidiocarps of *T. bambusina* could be collected at any season. As long as sufficient moisture is available for normal expansion of the basidiocarp and for epibasidia to extend past the hymenial area, basidiospores can be produced. According to Buller (1922), gelatinous fungi imbibe water in contrast to non-gelatinous forms which absorb water by capillarity. The gelatinous nature of basidiocarps in the genus *Tremella* seem to be an ecological adaptation for quick imbibition and retention of a water reserve (Ingold, 1959). The revival of dried specimens of *T. bambusina* when water is applied, is indeed very rapid, but the basidiocarp dries quickly as well. It would thus appear that most growth and development would take place in prolonged wet conditions.

From experiments in culture, the development of spore producing basidiocarps from mating time to spores could be well below 30 days.

The potential viability of the haploid cells in the dried condition has been measured as at least 18 days, and for basidiospores over 30 days.

SUMMARY

The tuberculate jelly fungus identified as *Tremella bambusina* Sacc. grows from the perithecia of *Valsa* and *Diaporthe* on species of higher plants. From 72 randomly selected, single spore isolates, compatibility tests indicated a "Tremella Type" tetrapolar mating system. A single pair of alleles at the "A" locus determine initiation and growth of conjugation tubes. Thirteen "B" factors controlling dikaryon formation, were identified. The latter were from seven basidiocarps, some of which grew within a few centimeters of each other on the same branch.

The yeast-like phase of this dimorphic fungus is easily grown in artificial media and growth can be measured by turbidimetric methods. Calcium compounds added to the medium resulted in better growth at higher temperatures but was not required for rapid growth at 15° to 20° C. Glucose, sucrose or maltose, but not sodium acetate, provided a carbon source for good growth. Ammonium compounds appeared to be utilized but nitrates were not. The vitamin thiamine is required. Good growth occurred over a pH range of 4.0 to 7.0, with the optimal level near pH 7.0

The conversion from budding to tube formation took place in the presence of conjugation hormones; conjugation appeared to be inhibited by an excess of the yeast phase. Dikaryotization took place in a manner previously described by Bandoni for *T. mesenterica*. Conjugation hormones did not

appear to be stable to autoclaving, and the formation of conjugation tubes was weak in liquid medium. Conjugation hormones were effective, laterally over 20 mm. of agar surface and behaved similarly to other *Tremella* hormones investigated. Conjugation tubes of opposite mating types were tropic when the tips approached within 10 μ. of one another on agar surfaces. Upon fusion, the dikaryon was established and a clamped hyphal stage grew through the agar medium. The best growth of conjugation tubes and the dikaryotic phase appeared along the outer margins of the inoculum. To insure good results from compatability tests, minimal amounts of inoculum had to be used. Optimal growth of the dikaryotic phase was found to be between the temperature range of 15-25° C.

Clamps with haustoria are common but simple clamps are typical of later stages of dikaryotic growth. There was no observable reaction when *T. bambusina* was grown with *Valsa* hyphae. Dedikaryotization occurred readily with the formation of the haploid stage.

Single spore isolates from basidiocarps found on different ascomycetes, were compatible and completed their life cycle in culture.

Spores germinate on the surface of the basidiocarp by repetition. Resultant spores were identical to the original basidiospore but smaller. Budding occurred in water and solid media while tubes were initiated on solid medium only in the presence of conjugation hormone(s).

Abnormal basidia, with branched or septate epibasidia and conidial formation were often found in cultures. Aborted probasidia were abundant on hyphae in agar and basidiocarps, but consistent spore production was not obtained. The transfer of the dikaryotic phase to new media resulted in rapid growth and, where the mycelium is exposed, formation of basidiocarps. Culture conditions affected the color, texture, size of the basidiocarp as well as the appearance of the hymenial surface. The variation of basidiocarp characteristics, basidiospore size and clamp connections suggest caution be used when these features are used for species delimitation.

The characteristics of *T. bambusina* make it a useful tool for future sexual, genetic and morphogenetic studies of basidiomycetes. The following appear worthy for future study.

1. Pair additional strains obtained from new collections of pyrenomycete inhabiting *Tremella*, with known types; as a means of increasing our knowledge on the distribution and number of "B" factors and for detecting new species.

2. Observe the reaction of spores to the environment of the hymenial surface to help in the understanding of the mechanism of repetition.

3. Investigate the effects of calcium on growth of the haploid phase at high temperatures.

4. Check the effects of sucrose, amino acids and tem-

perature on the interconversion of budding and hyphal growth.

5. Examine the influence of medium age, pH and aeration upon basidiocarp formation and sporulation.

6. Classify types of conidia, based upon close examination of their nuclear condition and method of formation.

7. Study the relationships existing between *T. bambusina* and *Valsa* and *Diaporthe*. *Tremella* stocks could be introduced in either gas sterilized perithecia or perithecia produced in culture by known species.

8. Ascertain the effects of autoinhibitors. Quantatative experiments on the inhibition of hyphal growth by the yeast phase could easily be made.

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APPENDIX A

Collections Studied and Isolated Strains of *Tremella bambusina* Sacc.

I. Collections

I examined 33 collections identified as *T. bambusina* (listed in Table XXI). All collections listed with SGB numbers were from fresh material, all others were from the private herbarium of Dr. R.J.Bandoni. I consider collections RJB611 and RJB1675 significantly different and they were not considered in the technical description.

II. Strains isolated

Of the 111 single spore isolates obtained and examined, only 72 were used. These were from seven collections as listed in Table XXII.

Isolate 101 was obtained from the only colony that appeared viable from over 40 spores isolated from collection SGB 619. Apparently high temperature in the incubator over one weekend caused death in all other isolates.

The 400 series isolates from collection RJB4041 were obtained by Dr. R.J.Bandoni and maintained in the U.B.C Mycological Culture Collection. Although the cultures were viable, the results of crosses with other isolates were inconsistent and weak.

There was no interaction between any isolates from collection SGB624. I believe they represented one mating type only. Isolate 501 appeared to be contaminated with another mating type because of the appearance of a few weakly

formed conjugation tubes in all crosses.

I made isolates from collections SGB625A, SGB625B, and SGB625C which represented three basidiocarps within 35 cm. of each other on the same shoot. Cultures 561 to 584 (SGB 625C) were made from the lowest basidiocarp, approximately 20 cm. from the middle basidiocarp (SGB625B). The isolates resulting from the lowest basidiocarp were extremely slow growing and the individual cells appeared larger and more spherical than all others (Fig. 62). I have observed no conjugation tubes either among these isolates or between isolates of this series and others. In culture they are identical in gross morphology to other cultures of *T. bambusina,* but appear to partially digest the agar surface. These isolates may represent a related species of *Tremella*. Four strains were grown on Niger seed medium (See Appendix B: Media) in a test for *Cryptococcus neoformans*, but the results were negative.

All other isolates were crossed and the calculated "A" and "B" factors were assigned from their interaction with strains 1, 2, 3, and 6 to which the mating types were arbitrarily assigned. These are listed in Table XXIII.

Table XXI. Collections Studied

Collection number	Place collected	Date collected	Collected upon	Associated with
RJB70	Vancouver, B.C.	11 Oct. 1958	*Rubus*	*Valsa*
RJB71	Vancouver	11 Oct. 1958	*Rubus spectabilis*	*Valsa*
RJB126	Vancouver	21 Dec. 1958	*Rubus spectabilis*	*Valsa*
RJB130	Vancouver	26 Dec. 1958	*Rubus spectabilis*	*Valsa ceratophora*
RJB186	Vancouver	25 Jan. 1959	*Salix*	*Diaporthe*
RJB299	West Vancouver, B.C.	4 Apr. 1959	Conifer	*Valsa*
RJB605	Vancouver	23 Sept 1959	*Rubus spectabilis*	*Valsa*
RJB608	Vancouver	25 Sept 1959	*Sambucus pubens*	Pyrenomycete
RJB611	Vancouver	25 Sept 1959	*Cornus nuttallii*	Pyrenomycete
RJB614	Vancouver	25 Sept 1959	*Tsuga heterophylla*	*Valsa abietis*
RJB781	Vancouver	21 Nov. 1959	*Acer circinatum*	*Valsa*
RJB906	Vancouver	2 Apr. 1960	*Tsuga heterophylla*	*Valsa*
RJB1307	Mt. Seymour, B.C.	14 June 1960	*Vaccinium ovilifolium*	Pyrenomycete
RJB1418	Vancouver	23 Oct. 1960	*Tsuga heterophylla*	*Valsa*
RJB1423	Vancouver	23 Oct. 1960	*Sambucus pubens*	Pyrenomycete
RJB1495	Vancouver	15 Jan. 1961	*Sambucus pubens*	*Diaporthe*
RJB1533	Vancouver	9 Feb. 1961	unknown	*Diaporthe*
RJB1536	Vancouver	9 Feb. 1961	*Sambucus pubens*	*Diaporthe*
RJB1537	Vancouver	9 Feb. 1961	*Cornus nuttallii*	*Diaporthe*
RJB1561	Squamish, B.C.	21 Feb. 1961	*Acer circinatum*	*Valsa clavigera*

Table XXI. continued

RJB1560	Squamish	25 Feb. 1961	*Acer circinatum*	*Valsa clavigera*
RJB1674	West Vancouver, B.C.	15 Apr. 1961	*Alnus rubra*	*Valsa*
RJB1675	West Vancouver	15 Apr. 1961	*Cornus nuttallii*	Pyrenomycete
RJB2786	Vancouver	5 Apr. 1963	*Cytissus scoparius*	*Gibberella?*
RJB4041	Vancouver	6 Dec. 1965	unknown	Pyrenomycete
SGB619	Vancouver	26 Nov. 1966	*Rubus spectabilis*	*Valsa*
SGB620	Vancouver	14 Jan. 1967	*Thuja plicata*	*Valsa*
SGB624	Manning Park, B.C.	11 May 1967	*Rubus*	*Valsa*
SGB625	Hope, B.C.	11 May 1967	*Rubus spectabilis*	*Valsa*
SGB645	Vancouver	23 Aug. 1968	*Cornus nuttallii*	*Diaporthe*
SGB655	Vancouver	19 Sept 1968	*Pseudotsuga menzisii*	*Valsa*
SGB658	Vancouver	20 Aug. 1969	*Tsuga heterophylla*	*Valsa*

III. Isolate numbers in culture

Table XXII. Numbers of isolates in culture

Collection number	Associated with	Isolate numbers assigned
SGB619	*Rubus, Valsa*	101
SGB620A	*Thuja, Valsa*	1, 2, 3, 4, 5, 6, 7.
B		8, 9, 10, 11, 12, 18.
C		13, 14, 15, 16, 17.
D		19, 20.
RJB4041	*Rubus, Valsa*	401, 402, 403, 404.
SGB624	*Rubus, Valsa*	501, 502, 503, 504, 505.
SGB625A	*Rubus, Valsa*	521, 522, 523, 524, 525, 527, 528, 529, 530, 531, 532, 533, 534, 535, 536, 537, 538.
B		541, 542, 543, 544, 545, 546, 547, 548.
C		561, 562, 563, 564, 565, 566, 567, 568, 569, 570, 571, 572, 573, 574, 575, 576, 577, 578, 579, 580, 581, 582, 584.
SGB645A	*Cornus, Diaporthe*	601, 602, 603, 604.
B		611, 612, 613, 614, 615, 616, 617, 618, 619, 620, 621, 622.
SGB655	*Pseudotsuga, Valsa*	701, 702, 703, 704, 705, 706, 707, 708, 709, 710, 711, 712, 713, 714, 715, 716, 717.

IV. Calculated "A" and "B" factors

Table XXIII. The calculated "A" and "B" genotypes and factors assigned to the strains isolated.

Collection SGB620A. Hosts, *Thuja plicata* and *Valsa*

AB_1 = 1.
AB_2 = 3, 4, 5.
aB_1 = 6, 7.
aB_2 = 2.

Collection SGB620B. Hosts, *Thuja plicata* and *Valsa*.

AB_3 = 12.
AB_4 = 8.
aB_3 = 9, 11.
aB_4 = 10, 18.

Collection SGB620C. Hosts, *Thuja plicata* and *Valsa*.

AB_5 = 14.
AB_6 = *
aB_5 = 13, 15.
aB_6 = 16, 17.

Collection SGB625A. Hosts *Rubus spectabilis* and *Valsa*.

AB_7 = 523, 524, 534, 535.
AB_8 = 527, 530, 533, 536, 537.
aB_7 = 531, 538.
aB_8 = 521, 522, 525, 528, 529, 532.

Collection SGB625B. Hosts, *Rubus spectabilis* and *Valsa*.

AB_9 = 542, 544, 545.
AB_{10} = 543.
aB_9 = 541, 547.
aB_{10} = 546, 548.

Table XXIII. continued

Collection SGB645. Hosts, *Cornus nuttallii* and *Diaporthe*.

AB_{11} = 618, 619, 622.
AB_{12} = 613, 614, 615.
aB_{11} = 612, 616, 617, 620, 621.
aB_{12} = 611.

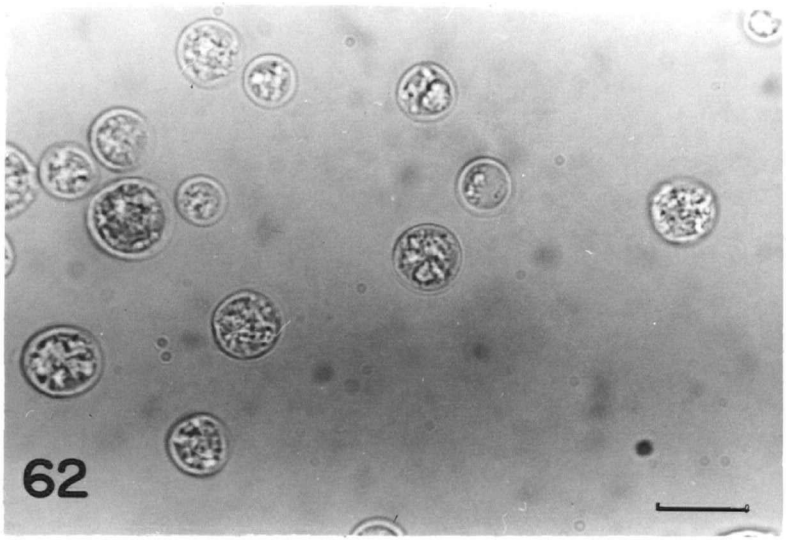
Collection SGB655. Hosts, *Pseudotsuga menziesii* and *Valsa*.

AB_{13} = 705, 710.
AB_{14} = 702, 703, 709, 712, 713, 714, 715.
aB_{13} = 701, 706, 717.
aB_{14} = 704, 707, 708, 711, 716.

Figure 62

Haploid phase (culture 561) obtained from collection SGB 625 C.

Fig. 62. Large round yeast-like cells typical of all isolates from the collection 625C.


62

APPENDIX B

Culture Media

(1) Synthetic Medium A, (SMA). (Basal synthetic medium, Lilly and Barnett, 1951).

Substance	Amount	
Glucose	10.0	g.
l-(+) asparagine	2.0	g.
potassium phosphate, monobasic. KH_2PO_4	1.0	g.
magnesium sulfate. $MgSO_4 \cdot 7H_2O$	0.5	g.
ferric sulfate. $FeSO_4 \cdot 7H_2O$	4.0	mg.
zinc sulfate. $ZnSO_4 \cdot 7H_2O$	4.0	mg.
manganous sulfate, monohydrate. $MnSO_4 \cdot 4H_2O$	4.0	mg.
thiamine hydrochloride	100	µg.
biotin	5	µg.
distilled H_2O	1	l.

pH of prepared medium = 4.5

(2) Synthetic Medium B, (SMB).

Substance	Amount	
Glucose	10.0	g.
ammonium sulfate. $(NH_4)_2SO_4$	2.0	g.
KH_2PO_4	1.0	g.
$MgSO_4 \cdot 7H_2O$	0.5	g.
calcium sulfate. $CaSO_4 \cdot 2H_2O$	1.0	g.
thiamine hydrochloride	100	µg.
biotin	5	µg.
Vogel's trace element solution (See item #5)	5	ml.
distilled H_2O	1	l.

pH of prepared medium = 3.3

(3) Synthetic Medium C, (SMC).

Substance	Amount	
glucose	10.0	g.
Ammonium tartrate. $(NH_4)_2C_4H_4O_6$	2.0	g.
KH_2PO_4	1.0	g.
$MgSO_4 \cdot 7H_2O$	0.5	g.
calcium nitrate. $Ca(NO_3)_2 \cdot 4H_2O$	2.0	g.
thiamine hydrochloride	100	µg.
biotin	5	µg.
Vogel's trace element solution	5	ml.
distilled water	1	l.

pH of prepared medium = 4.3

(4) Synthetic Medium D, (SMD).

glucose	10.0	g.
$(NH_4)_2C_4H_4O_6$	2.0	g.
KH_2PO_4	1.0	g.
$MgSO_4 \cdot 7H_2O$	0.5	g.
$Ca(NO_3)_2 \cdot 4H_2O$	1.0	g.
Microelement solution (Fe, Zn, Mn, Cu) (See item 6 below)	2.0	ml.
vitamin stock solution (Thiamine, biotin)	10.0	ml.
distilled water	1	l.

pH of prepared medium = 5.3

(5) Vogel's Trace Element Solution (Vogel, 1956)

Substance	Amount	
citric acid $H_3C_6H_5O_7 \cdot H_2O$	5.0	g.
$ZnSO_4 \cdot 7H_2O$	5.0	g.
ferrous ammonium sulfate. $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	1.0	g.
cupric sulfate. $CuSO_4 \cdot 5H_2O$	0.25	g.
$MnSO_4 \cdot 4H_2O$	0.05	g.
boric acid. H_3BO_4	0.05	g.
sodium molybdate. $Na_2MoO_4 \cdot 2H_2O$	0.05	g.
distilled H_2O	95	ml.
chloroform	1	ml.

Preparation: Substances dissolved in the order listed; stock solution kept refrigerated (not autoclaved)

(6) Microelement Solution (Fe, Zn, Mn, Cu)

Substance	Amount	
ferric nitrate. $Fe(NO_3)_3 \cdot 9H_2O$	181.0	mg.
$ZnSO_4 \cdot 7H_2O$	110.0	mg.
$MnSO_4 \cdot 4H_2O$	51.0	mg.
distilled H_2O	150	ml.

Elements dissolved in water and c.p. H_2SO_4 added until any precipitate dissolves. The substances below are then added.

$CuSO_4 \cdot 5H_2O$	110.0	mg.
distilled H_2O	100	ml.

Stock solution refrigerated (not autoclaved)

(7) Thiamine-Biotin Stock Solution

Substance	Amount	
thiamine	1.0	µg.
biotin	50.0	µg.
ethyl alcohol (40%)	100	ml.

Ten ml. stock solution provided 100 ug. thiamine and 5 ug. biotin per liter of medium

(8) Conjugation Medium, (CJA).

Substance	Amount	
glucose	2.0	g.
soytone	2.0	g.
agar	15	g.
distilled H_2O	1	l.

pH of prepared medium = 7.2

(9) Liquid Conjugation Medium, (CJL).

Same as CJA except agar was omitted

(10) Malt, Yeast, Soytone Agar Medium, (MYS).

Substance	Amount	
malt extract	15.0	g.
yeast extract	0.5	g.
soytone	2.5	g.
agar	15	g.
distilled H_2O	1	l.

pH of prepared medium = 6.2

(11) Niger Seed Medium, (NSM). For *Cryptococcus neoformans* identification. (Gordon, et al., 1965)

Substance	Amount	
glucose	10.0	g.
creatinine	1.0	g.
KH_2PO_4	1.0	g.
Niger seed (pulverized)	50	g.
agar	15	g.
distilled H_2O	1	l.

(12) Potato Dextrose Agar Medium (PDA). (Ainsworth, 1967)

Substance	Amount	
potatoes, peeled, cut-up	200	g.
glucose	10.0	g.
agar	15	g.
distilled water to	1	l.

Preparation: Potatoes boiled in 1 liter distilled water for 30 min. and solution drained into glucose, agar mixture. Distilled water added to boiled potatoes and drained again into container to make 1 liter.

(13) Potato Sucrose Agar Medium, (PSA). (Ainsworth, 1967)

Same as PDA except sucrose was substituted for glucose.

(14) Rolled Oat Agar, (ROA). (Bandoni, pers. comm.)

Substance	Amount	
rolled oat grains	5-6 grains/Petri plate	
agar	15	g.
distilled H_2O	1	l.

Preparation: Several oat grains were placed within a Petri plate and autoclaved at 121° (15 lbs. pressure) for 20 min. When cool, autoclaved water agar was poured over oats within dish.

All media were autoclaved at 15 lbs. pressure (121° C.) for 15 minutes unless otherwise stated. Glucose, maltose, soytone and agar were from Difco Labs. Major salts in the media were from J.T.Baker Co.

APPENDIX C

Stains and Solutions

(1) Congo Red. (Bandoni, pers. comm.)

Substance	Amount	
Congo Red	4.0	g.
distilled H_2O	100	ml.

(2) Phloxine. (Martin, 1934 as modified by Bandoni, pers. comm.)

Substance	Amount	
Phloxine	4.0	g.
distilled H_2O	100	ml.
formalin		

Stain was dissolved in water and a few drops of formalin were added as a preservative.

(3) Cleaning Solution.

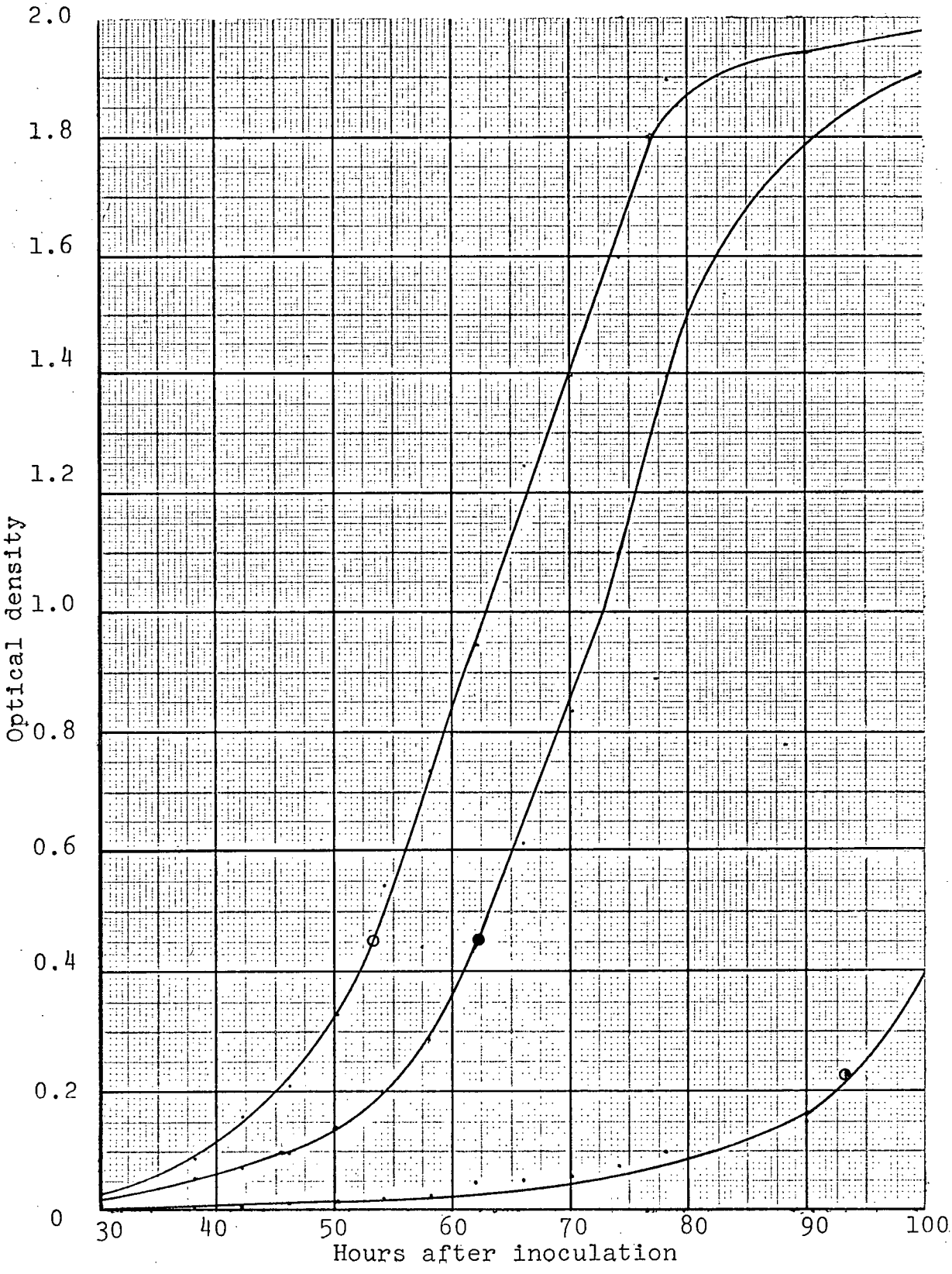
Substance	Amount	
potassium dichromate. $K_2Cr_2O_7$	60	g.
sulfuric acid, concentrated. H_2SO_4	460	ml.
distilled H_2O	300	ml.

APPENDIX D

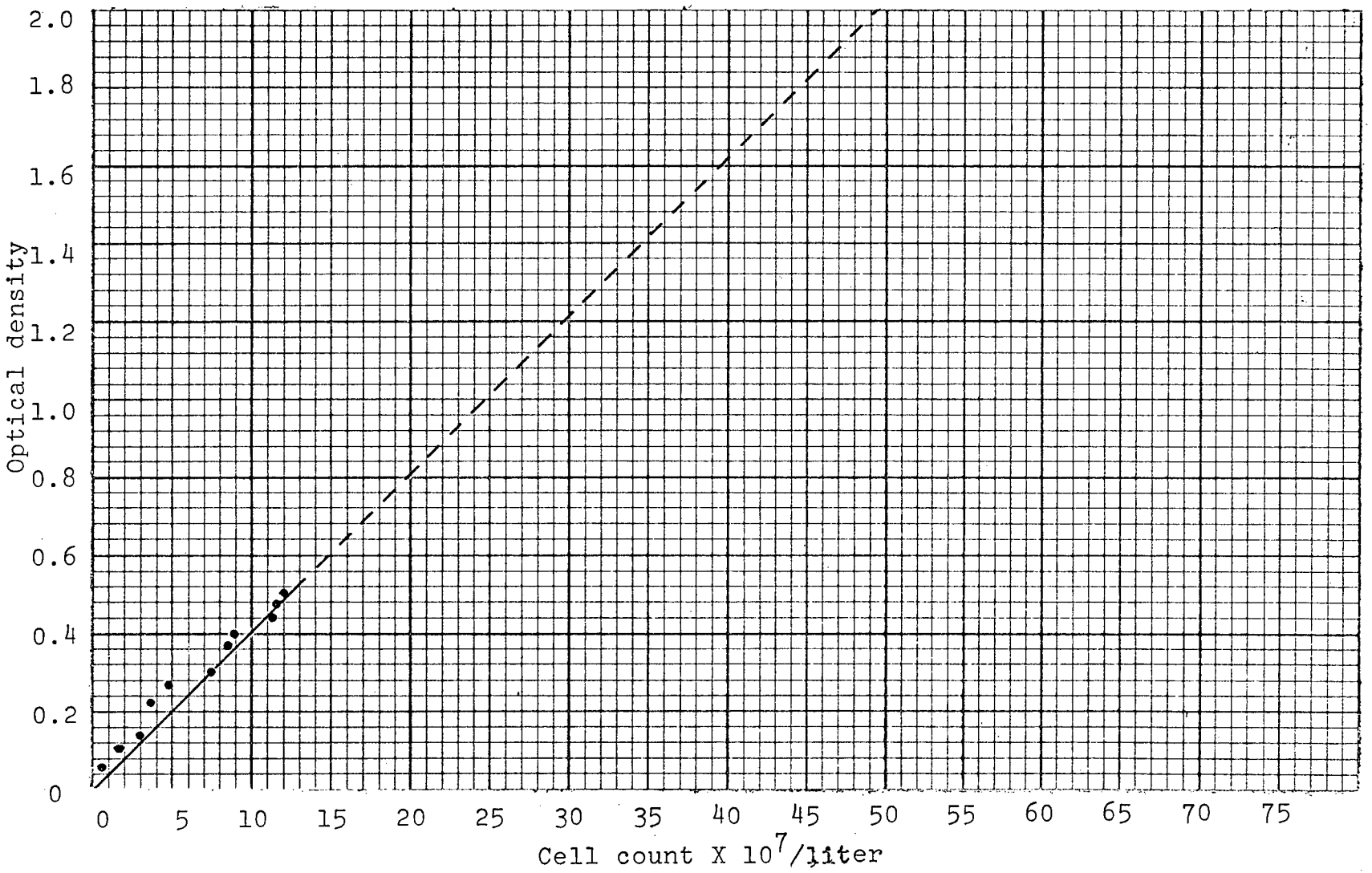
Growth Curve and Optical Density Charts

Graph 3. Growth curve of *T. bambusina* in SMD at 20° C.

	Strain number	Initial O.D.	Drops of inoculum
○	710	0.50	6
●	710	0.52	3
◐	12	0.35	3


2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Optical density
30
40
50
60
70
80
90
100
Hours after inoculation

Graph 4. Optical density compared with cell number of *T. bambusina*.


Optical density
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
Cell count X 10^7/liter