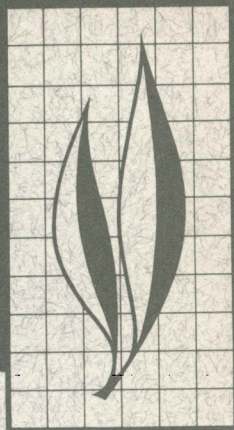


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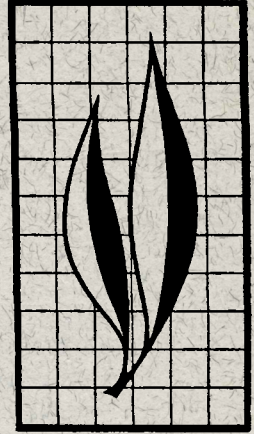
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Production of the Perfect Stage of *Mycena citricolor* (Berk. and Curt.) Sacc.

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The taxonomy and biology of *Mycena citricolor* (Berk. and Curt.) Sacc. is reviewed and techniques of induction of basidiocarps by certain other fungi are described.

Mycena citricolor was induced to produce the perfect stage when grown in co-culture with *Penicillium oxalicum*, *P. palitans*, *P. cyclopium*, *P. brevi-compactum*, or *P. viridicatum*. Basidiocarp yield was highest when *M. citricolor* was grown in co-culture with *P. oxalicum*. A low percentage of field isolates of *M. citricolor* were able to produce some basidiocarps in mono-culture, but yield was greatly increased when this fungus was grown in co-culture with *P. oxalicum*. Other isolates of *M. citricolor* produced basidiocarps only when grown in co-culture with *P. oxalicum*. However, several other basidiomycetes failed to produce the perfect stage when grown in co-culture with *P. oxalicum*.

A basidiocarp-stimulating substance(s) (BSS) was present in sterile supernatant fluids from cultures of *Penicillium oxalicum*. BSS resisted autoclaving and readily passed through dialysis tubing. Yield of BSS was highest when *P. oxalicum* was cultured in potato dextrose broth (PDB) or Emerson's YpSs medium in still culture for 1 week under laboratory conditions. *P. oxalicum* grown in PDB produced BSS independently of light conditions, but *M. citricolor* produced basidiocarps in response to BSS (or in co-culture with *P. oxalicum*) only when grown in alternating periods of light and dark. *M. citricolor* produced basidiocarps only when it was cultured on natural or semi-synthetic media.

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Production of the Perfect Stage of *Mycena citricolor* (Berk. and Curt.) Sacc.^{1,2}

INTRODUCTION

MYCENA CITRICOLOR (Berk. and Curt.) Sacc., a basidiomycete in the family Agaricaceae, is the causal agent of American leaf spot of coffee. This is probably the most important coffee disease in the Western Hemisphere, causing severe losses in several countries (Castaño, 1957; Fawcett, 1915; Perez, 1952; Sequeira, 1952; Uribe Arango, 1947; Wellman, 1961). Disease symptoms include necrotic lesions on the leaves, young stems, and berries, and premature defoliation caused by interruption of normal flow of auxins from leaf blade to petiole (Sequeira and Steeves, 1954). Infected berries also fall off the tree, and diseased young branches are easily broken by the wind (Carvajal, 1939; Sequeira, 1952).

The fungus produces two types of fruiting bodies: the gemmiferous stage (asexual), and the perfect stage (sexual). The gemmiferous stage consists of two parts: the stalk or pedicel, and the head, which was named a gemma by Buller (1934); gemmae also have been named "stilbum heads" (Maublanc and Rangel, 1914) or "stilboids" (Singer, 1949). However, gemmae are commonly known as "cabecitas" in Spanish (Echandi, 1956; Wellman, 1950). Gemmae are produced abundantly on the surface of coffee lesions, especially on leaves, and are the only known means of dissemination of the pathogen (Car-

vajal, 1939; Maublanc and Rangel, 1914; Wellman, 1950). Morphology of the gemmiferous stage has been studied in detail by Puttemans (1904) and Buller (1934). Good control of the disease is based on the inhibition of the formation of gemmae, thus breaking the life cycle of the pathogen (Bianchini *et al.*, 1958; Castaño, 1957; Diaz Moreno, 1961; Echandi, 1956; Echandi and Segall, 1958; Salas, 1960).

In addition to coffee, the fungus attacks several monocots, dicots, and pteridophytes. In some of these hosts the gemmiferous and sexual stages are produced, but in most of them only the gemmiferous stage has been observed. In a few hosts no reproductive structures are produced, and the inoculum is from other hosts that produce the gemmiferous stage (Buller, 1934; Carvajal, 1939; Fawcett, 1915; Sequeira, 1952 and 1958). Some hosts of the fungus are common weeds and shade trees in the coffee plantation.

The perfect stage was described for the first time by Maublanc and Rangel in 1914 as a small yellow mushroom (basidiocarp) about 1.0 to 1.5 cm in height. Subsequently, it has been reported a few times under natural conditions in coffee plantations, and in a few instances on culture media (Ashby, 1925; Buller, 1934; Sequeira, 1952 and 1954). The role of the basidiocarp of

¹ Portion of Ph.D. Thesis, by the Senior Author, University of California, Berkeley.

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M. citricolor in nature is not well understood, and studies under laboratory conditions have been hampered because of difficulty in obtaining the perfect stage.

The present studies were stimulated

by a chance observation that a *Penicillium* contaminant induced *M. citricolor* to produce basidiocarps in a medium that does not support its formation in monoculture.

TAXONOMY AND BIOLOGY OF *MYCENA CITRICOLOR* (BERK. AND CURT.) SACC.

This fungus has been known by many names. Those more commonly used are: *Stilbum flavidum* Cke., *Stilbella flavida* (Cke.) Kohl, *Agaricus citricolor* Berk. and Curt., *Omphalia flavida* (Cke.) Maubl. and Rangel, and, most recently, *Mycena citricolor*.

Sequeira (1952) discussed in detail the taxonomical aspect of this fungus and concluded that *M. citricolor* is the correct designation.

The asexual stage

The asexual or gemmiferous stage of the fungus was first described by Puttemans (1904), and later Buller (1934) expanded the description. The gemmiferous stage has been intensively studied, especially in relation to its importance as the means of dissemination of the pathogen (Bianchini *et al.*, 1958; Carvajal, 1939; Echandi, 1956; Wellman, 1950). It is produced abundantly on infected coffee leaves, stems and berries, and also on other hosts (Carvajal, 1939; Sequeira, 1952 and 1958).

The perfect stage

Basidiocarps of the fungus were first associated with the disease by Maublanc and Rangel (1914); they were obtained when infected leaves of *Eriobotrya japonica* (loquat), and some members of the melastomaceae and compositae, were maintained in moist chambers for several days and yielded the perfect and asexual stages of the fungus. The basidiocarps were described by Maublanc and Rangel (as translated and amended by Buller, 1934) as "Very minute, yellowish, pi-

leus thin, membranaceous, hemispheric-campanulate, depressed or subumbilicate in the centre, then more or less flattened, glabrous, radially striate, 1.5–2.5 mm in diameter, with an acute margin; stipe setiform, straight, thin, of the same colour, very minutely velvety, about 1–1.5 cm long."

Buller (1934) also made a complete study of morphological aspects of the basidiocarps. Carvajal (1939) was the first to find basidiocarps of the fungus growing under field conditions in association with the gemmiferous stage. He observed these structures on twelve herbaceous plants and four nonherbaceous plants, including coffee. Buller (1934) obtained the perfect stage on artificially inoculated leaves of *Bryophyllum calycinum*, *Nerium oleander*, and a species of *Ficus*.

Wellman (1961) obtained basidiocarps when naturally infected coffee leaves were maintained in a moist chamber. Dennis (1950) found basidiocarps on unidentified fallen leaves in a forest. In 1951, Castaño observed basidiocarps in Colombia on infected coffee leaves undergoing decomposition on the ground, and also on infected leaves maintained in a moist chamber. Quirós (1951) observed basidiocarps on infected coffee leaves and loquat. In 1952, Sequeira observed basidiocarps on fallen leaves of several hosts and also on infected leaves maintained under moist conditions. Barquero (1955) obtained basidiocarps when infected coffee leaves were kept in a moist chamber.

The perfect stage of the fungus has also been obtained on culture media. In

1925, Ashby isolated the fungus and obtained both the perfect and imperfect stages, thus proving by the pure culture method that both stages arise from the same mycelium; the two stages were produced on bread-water agar and cornmeal agar. Buller (1934) also reported the perfect stage on bread-water agar and oatmeal agar; he observed that some isolates of the fungus did not produce the perfect or the gemmiferous stage in culture.

Sequeira (1952, 1954) obtained basidiocarps on potato-dextrose agar, millet-seed agar, and unhusked rice-seed agar; on the last two media many basidiocarps were produced within 3 to 4 weeks, with about 10 per cent reaching maturity with the production of basidia.

Basidiospores

Maublanc and Rangel (1914), who first observed that *M. citricolor* was associated with a foliar disease of loquat, on which the perfect stage was produced, referred to the basidiospores as "... minute, ellipsoid or ovoid, apiculate below, hyaline, eguttulate or with one guttule, $4-5 \times 2.5-3 \mu$." (Buller translation, 1934).

Buller (1934) germinated basidiospores on a 2.5 per cent malt solution. He used basidiospores as inoculum by placing a pileus that was actively discharging spores over fresh wounds in a *Bryophyllum* leaf. No infection was obtained even in a moist chamber. Sequeira (1952) inoculated uninjured and injured coffee leaves with a light suspension of basidiospores, with no indication of infection. Due to the lack of sufficient studies on the infective capacity of the basidiospores, and because

the sporophores appear to be rare under natural conditions, the role of the perfect stage in the life cycle of the fungus is not clear.

Nuclear condition of the basidia and basidiospores

Sequeira (1952, 1954) studied the nuclear condition of the basidia and basidiospores of *M. citricolor* and found that the fungus is normally heterothallic, each basidiospore on germination giving rise to a primary mycelium with no clamp connections. However, in a few cases he observed sporadic formation of dikaryotic clamp-bearing mycelium of monosporus origin; apparently, spores are produced when two nuclei of different sex potential migrate into the basidiospores. Sequeira concluded that further genetic work is needed to obtain conclusive proof of the homo-heterothallism of this organism.

Nutrition in relation to reproduction of the fungus

No nutritional study has been reported in relation to the production of basidiocarps. In respect to the growth of the fungus, Sequeira (1952) studied the effect of carbon sources, nitrogen sources, vitamins, thiamine concentrations, and pH.

Echandi and Echandi (1958) studied the effect of 11 vitamins and 13 amino acids on the growth and gemmae production of *M. citricolor*; they determined that thiamine was required for gemmae production and most of the amino acids tested favored growth and gemmae production.

INDUCTION OF BASIDIOCARPS OF *MYCENA CITRICOLOR* BY OTHER FUNGI

The phenomenon of microorganisms inducing (or stimulating) the sporulation of fungi is well-known and several review articles have been written on this

subject (Porter and Carter, 1938; Raper 1952, 1960). Examples of this relationship have been observed in all four classes of fungi.

In Basidiomycetes, there are few reports of induction of the perfect stage by other microorganisms. In a series of papers Urayama (1957, 1960, 1965) reported stimulation of fruit-body formation of *Psilocybe panaeoliformis* by *Bacillus psilocybe* and *Bacillus* spp. He also observed a stimulatory effect on *Marasmius graminum*, *Agaricus bisporus*, *Coprinus radians*, *C. macrorrhizus* and *Stropharia* sp., and he partially purified the stimulant.

Leonard and Dick reported (1968) that *Hormodendrum cladosporioides* induced haploid fruiting bodies in *Schizophyllum commune*, and they determined some of the characteristics of the fruit-body-inducing substances.

Park and Agnihotri (1969) reported three bacteria as responsible for stimulation of abundant production of sporophores by *A. bisporus*. In the same year Hayes *et al.* reported that another bacterium induced the sporophore formation by *A. bisporus*, and that the multiplication of the bacterium is favored by metabolites of *A. bisporus*.

In most studies of induction of sporulation of organisms in dual culture, no

attempts have been made to determine the nature of the chemical substance(s) responsible for the morphogenic effects. However, in investigations on the light initiation of sexual stages, partially purified biologically active substances were obtained. In one study, a single inducing principle was obtained from members of all four classes of fungi. Trione *et al.* (1966) isolated and purified what they labelled P310 from 13 species of fungi belonging to Phycmycetes, Basidiomycetes, Ascomycetes, and Fungi Imperfecti. P310 was active in inducing sporulation of many unrelated fungi.

A *Penicillium* sp. (later identified as *P. oxalicum*) that appeared as a contaminant in a culture of *M. citricolor* growing on PDA induced *Mycena* to produce basidiocarps. The sexual stage is rarely produced by *M. citricolor* in pure culture. Basidiocarps were produced near the zone of contact between the two fungi. Because of the biological significance of this phenomenon, the interaction was examined more closely during our study, especially from the physiological point of view.

Materials and Methods

All *M. citricolor* isolates and those of the other fungi studied were maintained on potato dextrose agar (PDA) prepared as follows: 20 g of Difco® flake agar were enclosed in cheesecloth and washed overnight with tap water. The next morning the washed agar was rinsed with distilled water for 1 hour, after which the water was squeezed out. The moist agar was then placed in 500 ml of distilled water and autoclaved for 1 hour, or until the agar had melted. Two hundred and fifty grams of white potatoes (the variety "Klamath extra special" was used when available) were washed and sliced without peeling, placed in a container with 500 ml of distilled water, and simmered for an hour at 60°C. After heating the liquid

was decanted and 20 g of dextrose added to it. The melted agar was strained through cheese cloth and mixed with the potato broth. The volume was made up to 1 liter with distilled water and the pH of the solution adjusted to 6.4 with 1 N NaOH. All PDA was prepared in this way unless otherwise noted.

In studies on induction of basidiocarps of *M. citricolor* by other fungi, *M. citricolor* was grown on one side of regular deep Petri plates (9 cm diameter) on PDA (35 ml PDA per Petri plate), and the fungus to be tested as inducer was grown on the opposite side of the Petri plate. Inoculum of *M. citricolor* consisted of plugs obtained with a cork borer (0.5 cm diameter)

from *M. citricolor* colonies about 20-days-old growing on PDA. Cultures were exposed to normal diurnal light conditions in the laboratory.

In order to obtain a uniform fungal colony (especially of those that sporulate abundantly on culture media) each fungus was first transferred in a streak pattern (5 to 6 streaks per Petri plate) onto PDA. When good growth had occurred, but prior to sporulation, squares of 0.5 cm from the fungus colony were transferred onto PDA opposite to the *M. citricolor* inoculum—thus contamination of noncolonized areas of the agar surface was avoided. Depending on the rate of growth of the fungus, it was planted on the PDA in Petri plates in advance, at the same time or after *M. citricolor* was planted. The sequence was arranged so that a similar colony diameter of both interactants was obtained. For each fungus tested as an inducer, four replicates were used. The data, collected 1 month after both fungi were planted, consisted of the number of basidiocarps per replicate and an estimate of gemmae production rated with a scale of 0 to 4 (0 = no gemmae, 4 = numerous gemmae).

Many fungi were tested for their ability to induce the perfect stage of *M. citricolor* under the above conditions. The fungi included a wide variety of common soil fungi and plant pathogens (Salas, 1970).

Co-culture of *P. oxalicum* with *M. citricolor*

Most isolates of *M. citricolor* were obtained by the senior author on a trip made to Costa Rica in December, 1967. Most isolations were made from infected leaves, stems and berries of coffee (*Coffea arabica* L.) but a few were from *C. liberica* Bull. ex Hiern. A total of 65 *M. citricolor* isolates were made from the most important coffee-producing areas of Costa Rica. Five isolates of the fungus were obtained from infected cof-

fee leaves from Guatemala, and one isolate from Mexico was supplied by Dr. Jorge Galindo.

The 71 isolates of *M. citricolor* were grown alone and in co-culture with *P. oxalicum*. Four replicates of each isolate were grown alone, and four with *P. oxalicum* on PDA. One month after the initiation of the experiment, the number of basidiocarps, the gemmae production, and the production and germination of basidiospores, were determined for each *M. citricolor* isolate. Production of basidiospores was assessed by counting the number of spore prints on the water surface produced when 10 basidiocarps of each isolate were attached to the inside upper part of Petri plates containing sterilized distilled water. Once the basidiospores were collected on distilled water, they were transferred with the distilled water to test tubes and shaken for 3 minutes in a vortex test-tube shaker to disperse basidiospores in the water; the basidiospore suspension was then poured onto water-agar plates and germination of the basidiospores was determined after 48 hours.

Single-spore isolates of *P. oxalicum* were obtained by collecting spores with a transfer needle and immersing them in sterile distilled water containing Tween 20® (1 drop of Tween 20 per 15 ml of water) in a test tube and mixing the contents by use of a vortex test-tube mixer for 3 minutes. Spore concentration was then adjusted to about 100 per ml with a haemocytometer. The spore suspension was poured on water agar plates; after 24 hours good germination was obtained and single germinated spores were transferred to PDA slants. Each germinated spore was observed under dissecting and compound microscopes to insure that only one spore was transferred to the PDA slant. A total of 93 single-spore isolates of *P. oxalicum* was obtained; of these 50 were selected to determine their induction of

basidiocarps of *M. citricolor*. There was a small amount of variation in growth and sporulation in the 50 single-basidiospore isolates. Each one of the selected 50 single-spore *Penicillium* isolates and the original culture of *P. oxalicum* were tested against *M. citricolor* following the method already described.

Five replicates of each isolate were used. The final results on induction of basidiocarps and gemmae production of *M. citricolor* by each *P. oxalicum* isolate were obtained 1 month after initiation of the experiment.

Single basidiospore isolates of *M. citricolor* were obtained by collecting basidiospores from basidiocarps obtained when *M. citricolor* 143 was grown in co-culture with *P. oxalicum*. On water agar, the basidiospores started to germinate in 24 to 36 hours. Single germinated basidiospores observed under the stereoscopic and compound mi-

croscopes were transferred to PDA slants. One hundred fifteen basidiospore isolates were obtained from *M. citricolor* 143; when they were grown for 2 weeks, they could be divided into two groups. Group 1 included 99 isolates that produced a white mycelia, but no gemmae; group 2 included 16 isolates that produced gemmae. None of the 115 single basidiospore isolates produced basidiocarps on the PDA slants.

Of the 115 single basidiospore isolates, 50 were selected to determine their reaction when grown in co-culture with *P. oxalicum* (S-S-36). Thirty-seven of these isolates belonged to the first group (nongemmae producers), and 13 to the second group (gemmae producers). Five replicates were used for each combination. Data on the production of basidiocarps and gemmae (0 to 4 scale) were obtained 1½ months after initiation of the experiment.

Results

Of the several fungi tested (Salas, 1970) as possible inducers of the sexual structure of *M. citricolor*, those listed in table 1 were found to be inducers.

When the perfect stage of *M. citricolor* 143 was induced, the basidiocarps were produced near the zone where the two fungi made contact. Color plate I illustrates how this occurs with *P. oxalicum*. Production of gemmae by *M. citricolor* 143 was not influenced by co-culture with any of the tested fungi. Induction of basidiocarps of *M. citricolor* by four of the *Penicillium* inducers was tested after the isolates were maintained for 1½ years on PDA slants under laboratory conditions. The isolates were still active inducers, as shown in color plate II.

When 71 isolates of *M. citricolor* were grown in co-culture with *P. oxalicum*, all of them produced the perfect stage. Eleven isolates of *M. citricolor* produced basidiocarps when grown in monoculture; they included five isolates

from Guatemala and six isolates from Costa Rica. Of the 60 isolates that produced the perfect stage only in co-culture with *P. oxalicum*, twelve produced less than 20 basidiocarps per Petri plate; eighteen produced from 21 to 40 basidiocarps; fifteen produced 41 to 60 basidiocarps; six produced 61 to 80 basidiocarps; five produced 81 to 100 basidiocarps; and four produced more than 100 basidiocarps per replicate. Basidiospore production was abundant in 90 per cent of the isolates. Germination of basidiospores on water agar was between 90 and 95 per cent, and little variation was found among the different isolates.

In the eleven isolates that produced some basidiocarps when grown alone on PDA, the number of basidiocarps was increased three- or four-fold by co-culture with *P. oxalicum*. Production of gemmae by *M. citricolor* isolates ranged from low to high in monoculture and was not affected by co-culture with *P.*

TABLE 1
EFFECT OF INDUCER FUNGI ON BASIDIOCARP FORMATION
BY *MYCENA CITRICOLOR*

Fungus	Source	Average number of basidiocarps per replicate†
<i>Penicillium oxalicum</i> *	Laboratory contaminant	145.0
<i>P. palitans</i> *	Isolated from redwood seeds by J. N. Davidson	103.0
<i>P. cyclopium</i> *	Isolated from redwood seeds by J. N. Davidson	105.0
<i>P. brevi-compactum</i> *	Isolated from redwood seeds by J. N. Davidson	101.0
<i>P. viridicatum</i> *	Isolated from redwood seeds by J. N. Davidson	103.0
<i>Cladosporium</i> sp.	Laboratory contaminant	12.0
<i>Alternaria</i> sp.	Laboratory contaminant	11.0
<i>Alternaria</i> sp. (apparently <i>A. tenuis</i>)	Laboratory contaminant	0.8
<i>Phycomyces blakesteanus</i> + and - strains†		8.0

*Species identification by Dorothy I. Fennell, A.R.S. Culture Collection Investigations Fermentation Laboratory, Peoria, Illinois 61604.

† The cultures tested developed well-defined sectors with zygospores along the junctions of the (+) and (-) strains, but *M. citricolor* did not produce basidiocarps after 4 weeks; after 8 weeks, however, *M. citricolor* had overgrown the *Phycomyces* and produced basidiocarps, especially in the region of the (+) strain.

‡ Data collected after 1 month.

oxalicum. The number of basidiocarps produced by *M. citricolor* 143 when grown in co-culture with the 50 single-spore isolates of *P. oxalicum* ranged between 140 to 180 per replicate. The parent *P. oxalicum* isolate induced an average of 160 basidiocarps. Ten single-spore isolates induced more basidiocarps than the parent did—161 to 180; the other 40 single-spore isolates induced between 140 to 159. The best inducer-fungus was single-spore isolate number 36, which induced 180 basidiocarps per Petri plate. (This isolate was used in all further inducer studies with *P. oxalicum*.) The production of gemmae by *M. citricolor* 143 with the 50 single-spore isolates and the parent culture of

P. oxalicum was similar in all the cases.

Of the 50 single basidiospore isolates of *M. citricolor* grown in co-culture with *P. oxalicum* (S-S-36), 35 did not produce gemmae or basidiocarps, one isolate did not produce gemmae but did produce a few basidiocarps; four isolates produced some gemmae but no basidiocarps, and the remaining ten isolates produced gemmae and basidiocarps. The number of basidiocarps were considerably fewer than produced by the parent isolate. None of the other Basidiomycetes (including several agarics) grown in co-culture with *P. oxalicum* were induced to produce the sexual structure, and no other types of relationships were observed.

Discussion

M. citricolor, a plant-pathogenic basidiomycete, was induced to produce sexual structures when grown in co-culture with several other fungi. The most active inducer was *P. oxalicum*.

However, several other penicillia (*P. palitans*, *P. cyclopium*, *P. brevi-compactum*, and *P. viridicatum*) were shown to be good inducers, while a few other fungi (*Cladosporium* sp., two iso-

lates of *Alternaria* sp. and + and - strains of *P. blakesleanus*) stimulated some basidiocarp production.

Eleven of the 71 *M. citricolor* field isolates tested were capable of producing some basidiocarps when grown in monoculture, but the number of basidiocarps was increased greatly when grown in co-culture with *P. oxalicum*. The remaining 60 isolates produced the basidiocarps only when growing with *P. oxalicum*.

Many other fungi have been induced to produce reproductive structures when grown in co-culture with other organisms (see literature review on microbial stimulation of sporulation of fungi). This phenomenon has been reported for a few Basidiomycetes (Hayes *et al.*, 1969; Leonard and Dick, 1968; Park and Agnihotri, 1969; Urayama, 1957, 1960, 1965), although this is the first time that this reaction has been observed for *M. citricolor*.

The production of the sexual structures by *M. citricolor* in infected tissue has been reported rarely. In most cases the basidiocarps have been obtained when infected plant material was maintained in moist chambers (Barquero, 1955; Buller, 1934; Carvajal, 1939; Castaño, 1951; Maublanc and Rangel, 1914; Quirós, 1951; Sequeira, 1952), or in diseased tissues undergoing decomposition (Castaño, 1951; Carvajal, 1939; Dennis, 1950; Sequeira, 1952). Results of this study indicate that basidiocarp production by *M. citricolor* is dependent upon the activities of saprophytic fungi. However, differences in *M. citricolor* strains are certainly important because some field isolates used in this study were able to produce a few basidiocarps in monoculture.

Production of basidiocarps by *M. citricolor* in monoculture was first observed by Ashby (1925). Later, Buller (1934) obtained cultures from Ashby, and repeated his experiments, and also obtained sexual structures. More recently, Sequeira (1952, 1954) obtained

the perfect stage of *M. citricolor* in pure culture. He observed that only 10 per cent of the basidiocarps produced reached maturity and produced basidia. In our experiments, induced basidiocarps reached maturity and produced viable basidiospores.

Production of the perfect stage of *M. citricolor* under field conditions may be influenced by the presence of *M. citricolor* isolates genetically capable of producing the sexual stage, and by induction of the perfect stage by associated fungi. Extensive studies relating these results with the behavior of *M. citricolor* under field conditions are necessary before the importance of these factors can be assessed.

When single-spore isolates of *P. oxalicum* were tested for induction of the sexual stage of *M. citricolor*, no significant differences were observed. This indicates that the initial colony of *P. oxalicum* observed to induce the perfect stage of *M. citricolor* originated from a single spore.

When single basidiospore isolates of *M. citricolor* were tested against *P. oxalicum*, the great majority of the isolates that did not produce gemmae or basidiocarps were not induced to produce basidiocarps. Greater variation in basidiocarp induction was found in single basidiospore isolates than in field isolates. Only one investigator (Sequeira, 1952) has tried to infect coffee plants with basidiospores of *M. citricolor*, and another investigator (Buller, 1934) used another host—both with negative results—so it is not known if the sexual stage plays a role in pathogenesis. Because single basidiospore isolates often gave rise to cultures incapable of producing gemmae or basidiocarps in the presence or absence of inducer fungi, and because no experimental evidence exists that basidiospores can infect any plant, further pathogenicity tests with basidiospores of *M. citricolor* are needed before their role as inoculum can be assessed.

No other agarics, including three unidentified *Mycena* isolates, yielded the perfect stage when grown in co-culture with *P. oxalicum*. Even though only a few genera (six) in Agaricales were

tested, it appears that the phenomenon of induction by *P. oxalicum* is peculiar to *M. citricolor*. However, more fungal species in other classes should be tested before conclusions can be drawn.

Summary

Mycena citricolor (Berk. and Curt.) Sacc., a basidiomycete in the family Agaricaceae, which causes American leaf spot of coffee, was induced to produce its perfect stage when grown in co-culture with other fungi. When several common penicillia were tested, the best inducer was *Penicillium oxalicum*, followed by *P. palitans*, *P. cyclopium*, *P. brevi-compactum*, and *P. viridicatum*. Other fungi, such as *Cladosporium* sp., *Alternaria* sp. and *Phycomyces blakesleanus*, were weak inducers.

When 71 *M. citricolor* field isolates were tested for basidiocarp production, 11 were able to produce some basidiocarps in axenic culture. The number of

basidiocarps was greatly increased when grown in co-culture with *P. oxalicum*; the remaining 60 isolates produced the perfect stage only when in co-culture with *P. oxalicum*.

When single basidiocarp isolates of *M. citricolor* were grown in co-culture with *P. oxalicum*, the majority of them were not induced to produce basidiocarps; and, when grown in axenic culture, most of them did not produce the asexual stage (gemmae) and none produced basidiocarps.

None of the other basidiomycete species tested in co-culture with *P. oxalicum* was induced to produce basidiocarps.

INDUCTION OF BASIDIOCARPS OF *MYCENA CITRICOLOR* BY *PENICILLIUM OXALICUM*

The nutrition of some basidiomycetes growing in culture media was studied by several authors, and specific nutrient requirements for the production of basidiocarps were reported for a few of these fungi. A large amount of literature on growth and basidiocarp production was reviewed by Foster (1939), Steinberg (1939, 1950), Robbins and Kavanagh (1942), Hawker (1944), Robbins (1950), Fries (1950), Singer (1961), Taber (1966), and Volz and Beneka (1969).

In cases where the basidiocarp was induced by other microorganisms (Hayes *et al.*, 1969; Leonard and Dick, 1968; Park and Agnihotri, 1969; Urayama, 1957, 1960, 1965), little effort was made to study the effect of nutrients on these interactions. Urayama reported the induction of fruiting bodies

of *Psilocybe panaeoliformis* by certain bacteria and studied, among other factors, the influence of carbon and nitrogen sources and their ratios on the stimulative effect of the bacteria. Of the 14 carbon sources tested, sucrose and soluble starch allowed the greatest production of basidiocarps, while of the 19 organic nitrogen sources tested, the following allowed the greatest basidiocarp production: peptone, DL-valine, DL-serine, L-proline, DL-alanine and L-aspartic acid.

In *M. citricolor*, the effect of nutrients in relation to the production of basidiocarps has not been studied. The only reports of production of basidiocarps in culture media have been made using natural media, such as bread-water agar (Ashby, 1925; Buller, 1934), millet-seed agar and unhusked rice-seed agar

(Sequeira, 1952, 1954). A few studies on the effect of nutrition on mycelial growth and gemmae production of *M. citricolor* have been reported. Sequeira (1952) studied the effect of 15 carbon sources on mycelial growth and found that the best growth was obtained with starch, maltose, sucrose, and glucose as carbon sources; peptone was determined to be the best nitrogen source. Casein hydrolyzate, ammonium tartrate, and ammonium sulphate were less effective. Vitamins affected mycelial growth, with best growth obtained from a mixture of thiamine hydrochloride, riboflavine, pyridoxine hydrochloride, and calcium pantothenate. However, thiamine-HCl was the only vitamin that gave a striking increase in growth as compared with controls. The greatest mycelial growth

occurred when thiamine concentration was 1 mg per 40 ml of medium.

Echandi and Echandi (1958) studied the influence of vitamins and amino acids on growth and gemmae production of *M. citricolor*. They could not confirm Sequeira's work, as none of the 11 vitamins tested improved growth significantly. However, thiamine was required for gemmae production. All 13 amino acids tested favored growth of the fungus, and gemmae production was affected by L-tryptophane, L-valine, and L-asparagine.

The purpose of this study was to determine the effect of nutrition on production of basidiocarps by *M. citricolor* when grown in co-culture with *P. oxalicum*, and the effect of *P. oxalicum* culture fluids (CF) on *M. citricolor*.

Materials and Methods

M. citricolor isolate 143 and *P. oxalicum* single-spore isolate number 36 (*P. oxalicum* S-S-36) yielded the greatest number of basidiocarps in co-culture, and thus were selected for investigations on the effect of culture media on production of basidiocarps.

All glassware was washed with dichromate cleaning solution, rinsed overnight in running tap water, and re-rinsed five times with distilled water. Plastic Petri plates were used in all experiments. For experiments on the effect of natural, semi-synthetic and synthetic media on the production of basidiocarps by *M. citricolor* 143 when grown in co-culture with *P. oxalicum* S-S-36, 25 different media were used.

Five replicates of *M. citricolor* growing in pure culture and five replicates of *M. citricolor* and *P. oxalicum* growing in co-culture were used for each medium. Data on the number of basidiocarps and gemmae production for each treatment were taken 1 month after the initiation of the experiment. In one experiment various concentrations of thi-

amine-HCl and casein hydrolyzate replaced yeast extract in a modified YpSs medium. In another experiment with modified YpSs medium, the effect of various thiamine-HCl concentrations and a single concentration of casein hydrolyzate (2 g per liter) were tested.

Yeast extract contains several minor elements (Grant and Pramer, 1962). When yeast extract of YpSs was replaced with thiamine-HCl and casein hydrolyzate at different concentrations, the yield of basidiocarps by *M. citricolor* growing in co-culture with *P. oxalicum* was not as great as when yeast extract was used. Therefore an experiment was performed including minor elements in the best reconstructed medium, with thiamine-HCl (192 μ g per liter) and casein hydrolyzate (2 g per liter) replacing yeast extract in YpSs. The following minor element preparations were used: (A) Berthelot, 0.5 ml per liter, prepared as by Phillips (1968); (B) Murashige and Skoog, 10 ml per liter (Murashige and Skoog 1962); (C) Fowlks, Leben and Snell,

10 ml per liter (Fowlks *et al.*, 1967); and (D) Fe EDTA, 5 ml per liter.

Five media that supported the largest yield of basidiocarps in solid culture were selected for basidiocarp stimulating substance(s) production. Basidiocarp stimulating substance(s) (BSS) were estimated in these liquid media after the growth of *P. oxalicum* for 1, 2, 3, 4, 5 and 6 weeks. The five liquid media tested were:

1. PDB
2. Raper and Krongelb's medium (Raper and Krongelb, 1958)
3. YpSs (home made)
4. YpSs (home made) plus thiamine-HCl (192 μ g per liter) plus casein hydrolyzate (2 g per liter)
5. As 4, plus Berthelot's 0.5 ml per liter and Fe EDTA 5 mg per liter.

P. oxalicum was grown in 250 ml Erlenmeyer flasks containing 25 ml of media. *P. oxalicum* cultures were inoculated by collecting spores on a dry, sterile camel hair brush from a colony about 1 month old; at this stage the spores were dry and easy to collect in the bristles. The brush with the spores was then shaken inside the flask containing liquid culture medium. With this method, an abundance of spores were distributed on the surface of the medium. The cultures were kept under laboratory conditions, and after specified period the CF was separated from the mycelial mat. The liquid was then centrifuged for 10 minutes at 12,000 rpm and subsequently passed through 1.2, 0.45, and 0.22 μ Millipore® filters, which eliminated extraneous hyphae and spores. The filtered liquid from each culture was then divided in half and one portion was autoclaved for 20 minutes at 15 pounds. The pH of the CF of each culture was measured with a glass electrode every week when the samples were prepared.

The basidiocarp-inducing activity of each sample was bioassayed by placing

5 ml of the autoclaved or non-autoclaved liquid on 10-day-old colonies of *M. citricolor* (3.5 to 4.0 cm diameter) growing on PDA and YpSs. *M. citricolor* colonies were started near the border of the Petri plate, and the CF was placed in the part of the culture plate not covered by the *M. citricolor* colony.

Five replicates were prepared for each treatment, and the final results on the induction of *M. citricolor* basidiocarps were recorded 1 month after the initiation of each experiment. After they were washed several times with distilled water to eliminate any contaminating media, the inducing activity of mycelial mats of *P. oxalicum* was determined. Potato dextrose broth was added in the proportion of 1 ml per g of mycelial mat, and the mixture was macerated in a Waring blender for 10 minutes. The homogenate was centrifuged for 10 minutes at 12,000 rpm and the supernatant liquid passed through 1.2, 0.45 and 0.22 μ Millipore filters. Activity of the solutions was as described with the original CF.

The inducing effect of the supernatant liquid and mycelial mat extracts from *P. oxalicum* culture was tested when the fungus was grown for 1 week under the following conditions: (i) still culture under laboratory conditions and (ii) shaken culture under laboratory conditions (30 strokes per minute).

The effect of light on basidiocarp formation was tested when *M. citricolor* and *P. oxalicum* were co-cultured under (i) diurnal laboratory conditions (50 to 60 foot-candles from artificial and indirect sunlight), and (ii) continuous darkness. When the effect of light on the basidiocarp-inducing capacity of *P. oxalicum* CF was studied, *P. oxalicum* was grown on PDB in still culture for 1 week as follows: (i) diurnal laboratory conditions (50 to 60 foot-candles), (ii) continuous artificial (flu-

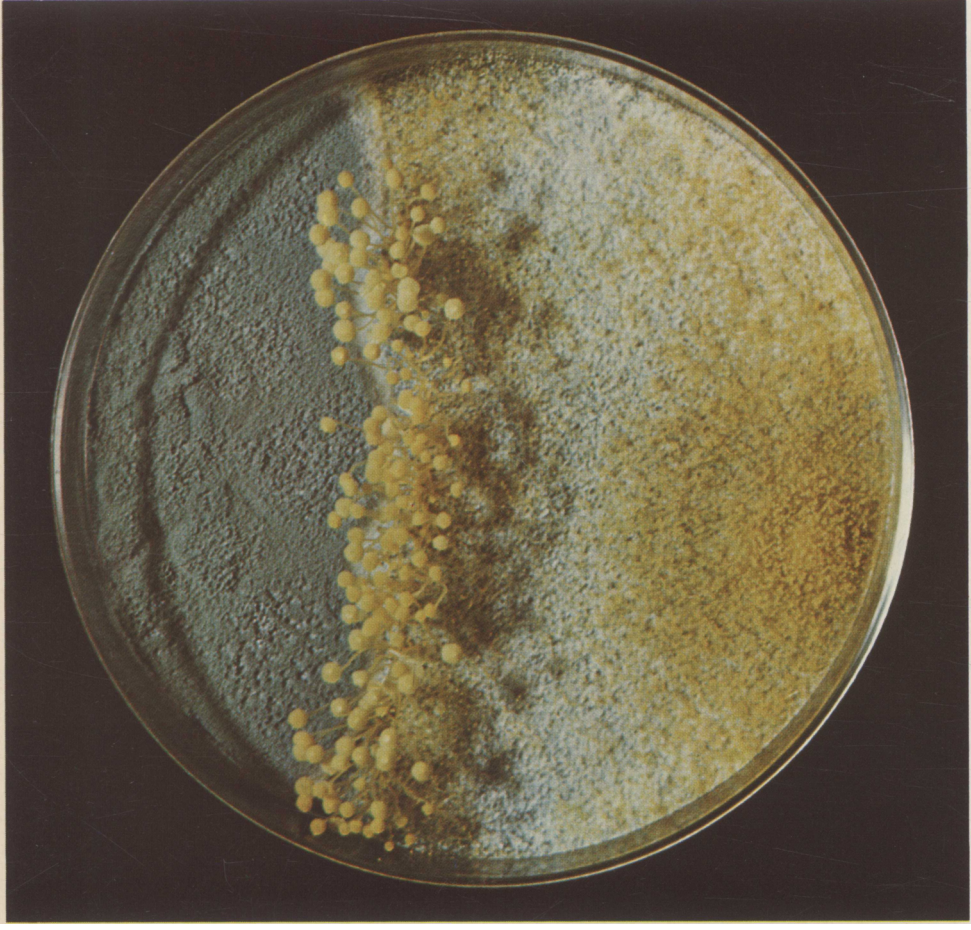


Plate I. Induction of basidiocarps of *Mycena citricolor* grown in co-culture with *Penicillium oraticum*.

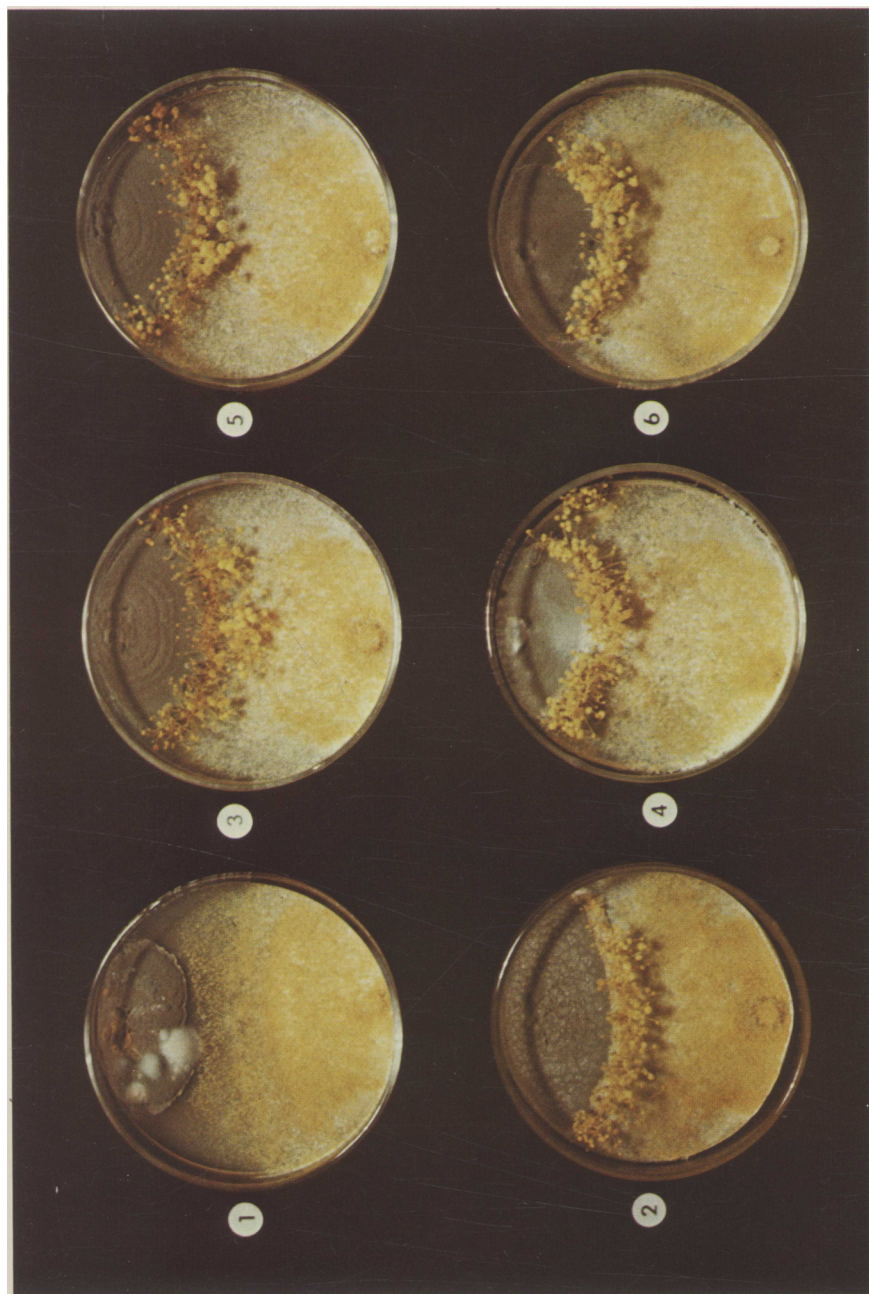


Plate II. Co-culture of *Mycena citricolor* with *Penicillium* spp. showing induction of basidiospores of *M. citricolor*. 1: *Penicillium* sp. (non-inducer); 2: *P. oxalicum*; 3: *P. cyclospium*; 4: *P. viridicatum*; 5: *P. cyclospium*; 6: *P. patitans*.

orescent) light (300 foot-candles), and (iii) continuous darkness. *M. citricolor* was grown on YpSs under these same

conditions. The CF obtained from *P. oxalicum* cultures was tested on *M. citricolor* in nine combinations.

Results and Discussion

Several different media supported the production of basidiocarps and gemmae by *M. citricolor* in co-culture with *P. oxalicum* (table 2). However, the basidiocarp yield on PDA and YpSs was substantially greater than on other media.

Thiamine and casein hydrolyzate partially replaced the yeast extract portion of YpSs (table 3); but if either of these materials were omitted, basidiocarps were not produced in co-cultures. Basidiocarp yield was not greatly effected by varying initial thiamine concentrations when initial casein hydrolyzate concentrations were held constant (table 4).

Yields of basidiocarps did not improve when minor element solutions were added to the modified YpSs in which yeast extract was replaced with

TABLE 2
PRODUCTION OF BASIDIOCARPS AND GEMMAE BY *MYCENA CITRICOLOR* IN CO-CULTURE WITH *PENICILLIUM OXALICUM*

Media	Number of basidiocarps produced	Gemmae production
PDA.....	175	3.5
YpSs (Difco®).....	141	4.0
V-8 juice agar.....	41	2.0
Raper and Krongelb's.....	40	4.0
Oat seed agar.....	20	2.0
Bille-Hansen's.....	18	1.0
Bread water agar.....	12	1.0
Sorghum seed agar.....	7	2.0
Difco® PDA.....	5	1.0
Madelin's.....	5	1.0
Difco®-corn meal agar.....	3	2.0
Rice seed agar.....	2	1.0
Difco® prune agar.....	2	2.0

TABLE 3

PRODUCTION OF BASIDIOCARPS AND GEMMAE BY *MYCENA CITRICOLOR* IN CO-CULTURE WITH *PENICILLIUM OXALICUM* ON A MODIFIED YpSs MEDIUM

Media	Number of basidiocarps produced	Gemmae produced
PDA.....	157	4.0
YpSs (Difco®).....	125	4.0
YpSs (home made).....	117	4.0
YpSs without yeast extract.....	0	0
Yeast extract in YpSs substituted by thiamine-HCl and casein hydrolyzate:		
48 µg/liter and 2 g/liter, respectively.....	73	1.0
12 µg/liter and 2 g/liter, respectively.....	35	1.0
24 µg/liter and 4 g/liter, respectively.....	29	1.0
96 µg/liter and 4 g/liter, respectively.....	27	1.0
Yeast extract in YpSs substituted by thiamine		
12 µg/liter.....	0	1.0
Yeast extract in YpSs substituted by casein hydrolyzate 2 g/liter.....	0	0

TABLE 4

YIELD OF BASIDIocarps AND GEMMAE WHEN *MYCENA CITRICOLOR* WAS GROWN IN CO-CULTURE WITH *PENICILLIUM OXALICUM* IN THE MODIFIED YpSs WITH DIFFERENT CONCENTRATIONS OF THIAMINE AND ONE CONCENTRATION OF CASEIN HYDROLYZATE REPLACING YEAST EXTRACT

Media	Number of basidiocarps produced	Gemmae produced
PDA.....	158	3.5
YpSs (Difco®).....	130	4.0
YpSs (home made).....	121	4.0
YpSs yeast extract replaced with thiamine-HCl and casein hydrolyzate:		
192 µg/liter and 2 g/liter, respectively.....	85	1.0
48 µg/liter and 2 g/liter, respectively.....	75	1.0
84 µg/liter and 2 g/liter, respectively.....	72	1.0
96 µg/liter and 2 g/liter, respectively.....	69	1.0
60 µg/liter and 2 g/liter, respectively.....	63	1.0
72 µg/liter and 2 g/liter, respectively.....	55	1.0
12 µg/liter and 2 g/liter, respectively.....	53	1.0
24 µg/liter and 2 g/liter, respectively.....	45	1.0
YpSs yeast extract replaced with casein hydrolyzate 2 g/liter.....	3*	0

* Apparently abnormal basidiocarps with long stalks and small caps.

thiamine and casein hydrolyzates (table 5). In fact, minor element solutions usually suppressed basidiocarp yields slightly. When *M. citricolor* 143 was grown alone no basidiocarps were produced on any medium, but the gemmae

production was similar to that of *M. citricolor* grown in co-culture with *P. oxalicum*.

Because of data obtained from co-culture on agar media, five media were selected to establish the kinetics of pro-

TABLE 5

YIELD OF BASIDIocarps AND GEMMAE WHEN *MYCENA CITRICOLOR* WAS GROWN IN CO-CULTURE WITH *PENICILLIUM OXALICUM* IN MODIFIED YpSs MEDIA

Media	Number of basidiocarps produced	Gemmae produced
PDA.....	167	3.5
YpSs (Difco®).....	128	4.0
YpSs (home made).....	124	4.0
Basal medium* plus:		
Bertheloth's + Fe EDTA.....	80	1.0
Bertheloth's solution.....	69	2.0
Murashige & Skoog's solution.....	68	2.5
Murashige & Skoog's solution plus Fe EDTA.....	66	2.5
Fowls <i>et al.</i> solution.....	56	3.0
Fe EDTA.....	43	1.0

* Yeast extract of YpSs substituted by thiamine-HCl (192µg/liter) and casein hydrolyzate (2 g/liter).

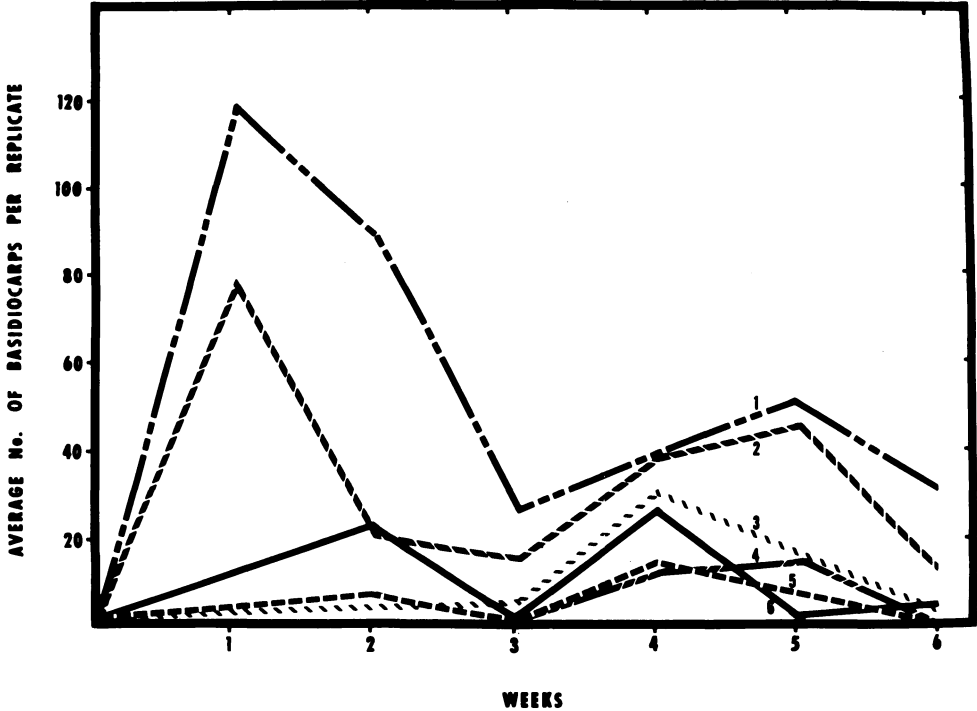


Fig. 1. Induction of basidiocarps of *Mycena citricolor* 143 with culture fluids from cultures of *Penicillium oxalicum* S-S-36 grown on: (1) PDB with *M. citricolor* growing on YpSs; (2) PDB with *M. citricolor* growing on PDA; (3) Raper and Krongelb's medium with *M. citricolor* growing on YpSs; (4) Raper and Krongelb's medium with *M. citricolor* growing on PDA; (5) YpSs with *M. citricolor* growing on PDA; and (6) YpSs with *M. citricolor* growing on YpSs.

duction of the basidiocarp-inducing substance(s) (BSS) by *P. oxalicum*. The best induction was obtained with the CF of *P. oxalicum* grown for 1 week on PDB, and the CF tested on *M. citricolor* colonies growing on YpSs (fig. 1). Activity of the CF was not affected by autoclaving. The second medium for the production of BSS was liquid YpSs, but its inducing activity was far lower than that of PDB. When the basidiocarp-inducing activity of CF was determined on *M. citricolor* growing on PDA or YpSs, it was observed that basidiocarps were produced on the *Mycena* colony that developed after BSS was added and not in the initial colony (fig. 2). This indicates that either BSS is not translocated to the old colony or that it cannot respond to

BSS. Nevertheless, only young mycelium can utilize it for the production of the basidiocarps. Of the other three liquid media tested, Raper and Krongelb's medium showed weak activity and the activities of cultures with the other two media was extremely low, averaging no more than three basidiocarps per replicate. When these three media were solidified with agar numerous basidiocarps were produced by *M. citricolor* in co-culture with *P. oxalicum*, but when *P. oxalicum* was grown on them in liquid form the activity of the CF was low.

Extracts of *P. oxalicum* mycelium grown on the five liquid cultures contained almost no BSS activity, indicating that most of the BSS was secreted into the medium. Figure 3

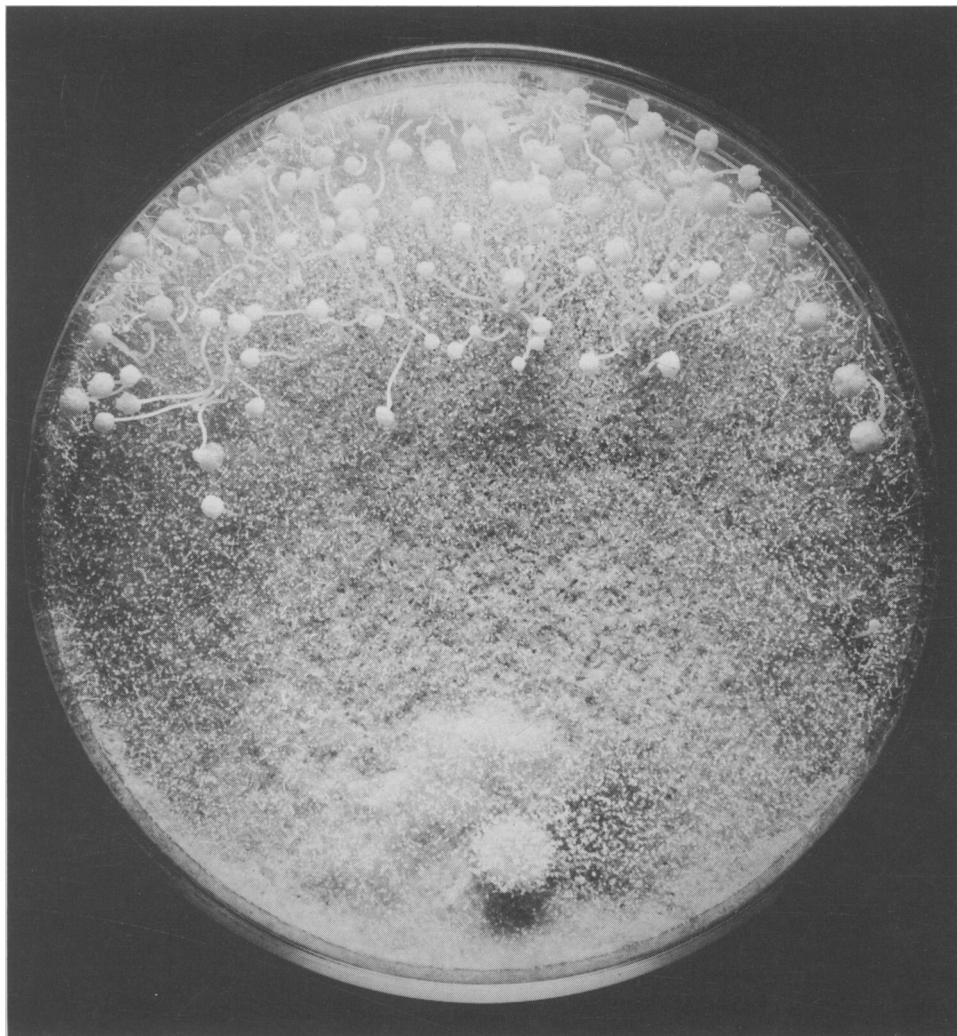


Fig. 2. Induction of basidiocarps of *Mycena citricolor* growing on YpSs by culture fluids of *Penicillium oxalicum* grown for 1 week on PDB on still culture. The basidiocarps were produced only in the *Mycena* mycelium developed after culture fluids were added and not in the initial colony. Basidiocarps were produced only when cultures were exposed to diurnal conditions.

shows the changes in pH in the autoclaved CF from the five culture media. The pH of the non-autoclaved media were similar to those autoclaved with a difference of ± 0.3 .

Induction of basidiocarps was best when *P. oxalicum* was grown for 1 week on PDB. At this stage the pH of the CF was 8.3. A similar pH value oc-

curred after growing *P. oxalicum* for 2 weeks on YpSs, but induction of basidiocarps with this material was about ten-fold less. These results seem to indicate that the pH is not correlated with the inducing activity of the CF.

No inducing activity was detected in the CF or the mycelia when *P. oxalicum* S-S-36 cultures were shaken nor

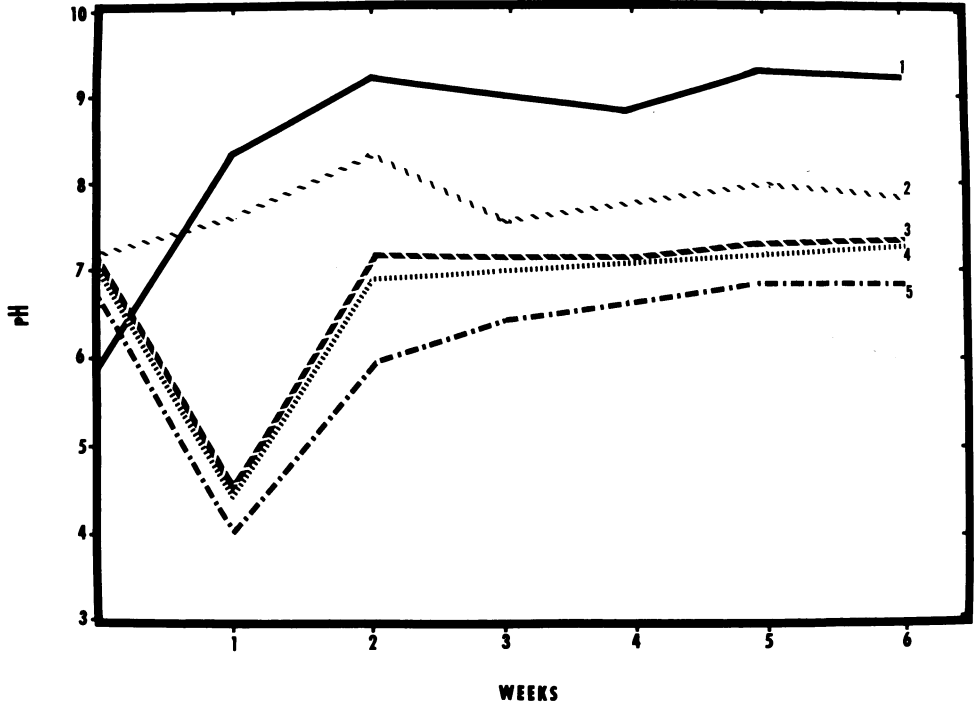


Fig. 3. pH values of liquid supernatant of *Penicillium oxalicum* S-S-36 grown in the following liquid media: (1) PDB; (2) YpSs; (3) modified YpSs yeast extract substituted by casein hydrolyzate (2 g/liter) and thiamine (192 µg/liter); (4) Raper and Krongelb's; and (5) same as 3 but Bertheloth's (0.5 ml/liter) and Fe EDTA (5 ml/liter) added.

from the mycelial mat from cultures grown under standing conditions. However, activity was present in the CF from cultures grown in still culture. Therefore, *P. oxalicum* was routinely grown in the laboratory in still culture.

Only co-cultures exposed to light resulted in basidiocarp production (table 6). When cultures maintained under dark conditions for 1 month were transferred to laboratory conditions, after 2 weeks an average of 30 basidiocarps per replicate were produced in co-culture. Moreover, an average gemmae production with an index of 2 was recorded when *M. citricolor* was grown in monoculture or in co-culture with *P. oxalicum*. These results agree with the reports (Buller, 1934; Sequeira, 1952; Rodriguez, 1964; Rodriguez and Arny, 1967) that light is necessary for gemmae production.

Results also corroborate those ob-

tained when *P. oxalicum* was grown in liquid culture under laboratory, dark, and continuous light conditions. The CF tested for induction of basidiocarps by *M. citricolor* indicated that *P. oxalicum* produced BSS in roughly equal quantities regardless of the light condition, whereas basidiocarps were produced by *Mycena* only when cultures were exposed to the diurnal conditions of the laboratory (table 7).

Nutritional requirements for production of the sexual structures of most basidiomycetes have not been established yet, but stimulation of fruiting in co-culture has recently been found to be a common phenomenon. Because fertility is expressed when external conditions are altered, competition for nutrients and pH changes in the medium are means by which basidiocarp production is induced (Cochrane, 1958). The classical studies of Klebs (1898, 1899,

TABLE 6

BASIDIOCARPS AND GEMMAE OF *MYCENA CITRICOLOR* OBTAINED WHEN THIS FUNGUS WAS GROWN ALONE AND IN CO-CULTURE WITH *PENICILLIUM OXALICUM* UNDER TWO LIGHT CONDITIONS

Fungi grown	Light conditions	Number of basidiocarps produced	Number of gemmae produced
<i>M. citricolor</i> and <i>P. oxalicum</i>	Laboratory conditions	123	3.0
<i>M. citricolor</i>	Laboratory conditions	0	3.0
<i>M. citricolor</i> and <i>P. oxalicum</i>	Dark conditions	0	0
<i>M. citricolor</i>	Dark conditions	0	0

1900) emphasized the important effect of external conditions on reproduction. Although Kleb's studies were with asexual structures, his views apply to sexual reproduction. He considered that reproduction was favored by a more limited range of environmental conditions than those that favor vegetative growth. On the basis of modern studies, Hawker (1966) noted that "... nutrition plays an important part in initiating the reproductive phase and in the development and maturation of spores and sporophores . . ." The molecular basis for the initiation of the reproductive phase in fungi is a topic of general interest currently.

The production of the sexual stage of *M. citricolor* has been reported only on natural media (Ashby, 1925; Buller, 1934; Sequeira, 1952, 1954). Although a few strains were found to fruit in monoculture on complex media in this study, this was unusual and few ba-

sidiocarps were produced. Basidiocarp production was affected by the composition of culture media. It was found in replacement media of YpSs that thiamine and complete amino acid solutions were required for high basidiocarp yields in co-culture. It is not clear, however, whether this is a direct nutritional effect on *M. citricolor* or indirectly via BSS synthesis by *P. oxalicum*. Because the BSS-synthesizing capacity of *P. oxalicum* is strongly influenced by culture media, speculation about the effects of nutrients on *M. citricolor* would be misleading. Once highly purified BSS is available, these types of studies will be simplified. The laboriousness of the bio-assay and impurity of BSS discourages detailed investigations on nutrition. Studies on the biochemical mechanisms involved in the initiation of *Mycena* basidiocarps and detailed genetic investigations also await further purification and characterization of BSS.

TABLE 7

NUMBER OF BASIDIOCARPS INDUCED ON *MYCENA CITRICOLOR* 143 BY CULTURE FILTRATES OF *PENICILLIUM OXALICUM* WHEN EACH FUNGUS WAS GROWN UNDER ONE OF THREE LIGHT CONDITIONS

Culture filtrates obtained from <i>P. oxalicum</i> grown under:	Number of basidiocarps induced on <i>M. citricolor</i> grown under:		
	Laboratory conditions	Constant light	Darkness
Laboratory conditions.....	90	0	0
Constant light.....	96	0	0
Darkness.....	93	0	0

Summary

Several natural, semi-synthetic, and synthetic media were tested in the induction of basidiocarps of *M. citricolor* grown in co-culture with *P. oxalicum* and the best results were obtained with freshly prepared PDA and Emerson's YpSs.

The basidiocarp-stimulating substance (BSS) was present in the sterile culture fluid from *P. oxalicum* still cultures. The highest inducing activity was obtained when *P. oxalicum* was grown in PDB under laboratory condi-

tions for one week. No BSS was detected in culture fluids from *P. oxalicum* shake cultures or in extracts from the mycelial mat of *P. oxalicum* grown in still culture. The inducing activity of the *P. oxalicum* culture fluid resisted autoclaving for 20 minutes at 15 psi.

Basidiocarps were produced by *M. citricolor* only when cultures were exposed to diurnal light conditions. Light had no effect on the production of BSS by *P. oxalicum*.

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