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VARIETAL CERATOCYSTIS MINOR IDENTIFIED FROM MYCANGIUM OF *DENDROCTONUS FRONTALIS*[1])

by

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ABSTRACT

The mycangium of the adult female southern pine beetle is a prothoracic glandular repository that serves for the propagation, transmission, and dissemination of fungi between and within host conifers. One fungus (SJB 133) was observed as reproducing amerosporous cells in the mycangium, but in larval galleries it developed a sporodochium-like layer of sympodulate conidiophores and conidia. Anascigerous reproduction in the galleries appeared to be stimulated by the presence of beetle larvae. The fungus exhibited many of the characteristics of ambrosial fungi associated with xylomycetophagous scolytids, thus indicating a possible analogous nutritional relationship. Inoculum from both the mycangium and beetle galleries produced an anascigerous *Sporothrix* sp. on various media. After prolonged development on potatoglucose agar the fungus produced a *Ceratocystis* ascigerous form. Virulence and serological studies resulted in the conclusion that the mycangial fungus (SJB 133) is a variety of *Ceratocystis minor*. A detailed description of the fungus is presented. Significance of the maintenance of the *Sporothrix* form in the mycangium and galleries is discussed.

INTRODUCTION

Phloem-inhabiting beetles of the genus *Dendroctonus*, in association with symbiotic fungi, cause destruction of conifers through endemic infestations and catastrophic epidemics throughout the United States and other parts of the world. Several of the most specialized species possess invaginations on the anterior entothoracic fold in the female prothorax. During beetle attack, fungi are transmitted to host trees in these structures, termed mycangia. The developing progeny feed in phloem colonized by the transmitted fungi (FRANCKE-GROSMANN, 1967).

Two fungi are prevalent in the mycangium of the southern pine beetle (*Dendroctonus frontalis* ZIMM.) (BARRAS & PERRY, 1972). One of these, isolate SJB 122, is a Basidiomycete presently under study by mycologists. The other, SJB 133, resembles both *Raffaelea* ARX &HENEBERT and *Sporothrix* HEKTON & PERKINS and appears related to an anascigerous form of *Ceratocystis* ELLIS & HALSTED.

This paper describes the appearance of SJB 133 in both the beetle mycangium and host phloem, and presents cultural and immunological studies that further delimit its taxonomic position.

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Materials and Methods

Characteristics of SJB 133 in the Mycangium

The generally tubular mycangium is bilaterally symmetrical about the prothorax of the female adult. The two lateral portions, each $\pm$ 100 μ in diameter, are joined by a narrower portion $\pm$ 10 μ in diameter at the dorsum of the pronotum. A right lateral view of the mycangium is shown in Fig. 1. Previous examination of over 500 mycangia revealed the presence of two filamentous fungi, SJB 133 and the Basidiomycete SJB 122. However, the two were never intermixed (Barras & Perry, 1972). Either a single species occupied the whole mycangium, or the two species occurred on opposite sides of the structure. In each case they grew as discrete masses of cells. This interesting phenomenon occurred in spite of the apparent receptivity of the whole mycangium to both fungi. Perhaps interspecific antagonism or selective secretions from the mycangial gland cells prevent intermixed growth. The constriction of the mycangium does not appear to present a physical barrier, for growth has been observed in this smaller portion also.

In thin sections and fresh preparations the fungus SJB 133 was observed as a mass of amerosporous cells (Fig. 2) exhibiting various shapes such as subglobose, obovate, clavate, and elongate (Fig. 3).

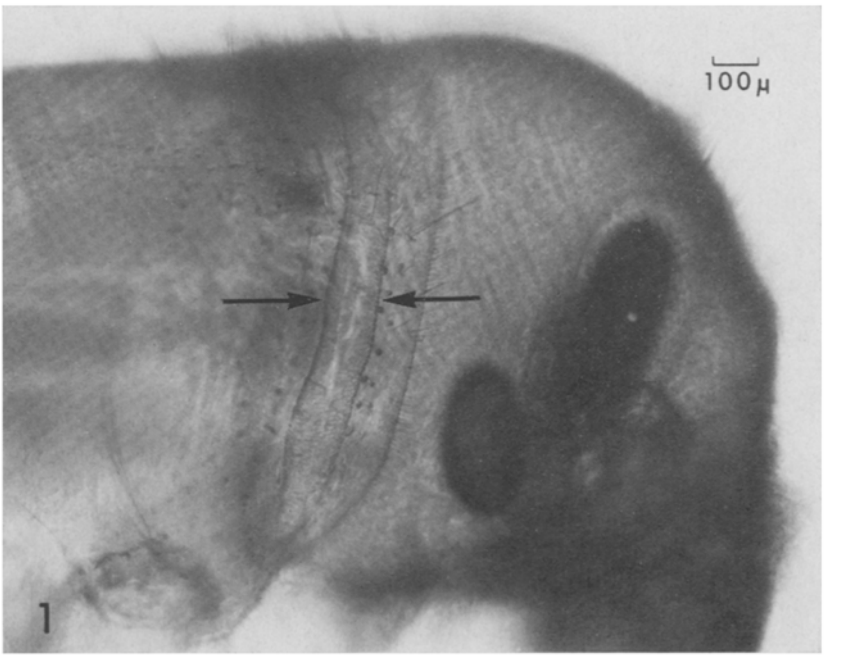

Fig. 1. Lateral view of female southern pine beetle (*Dendroctonus frontalis*) cleared in lactophenol to show mycangium (between arrows).

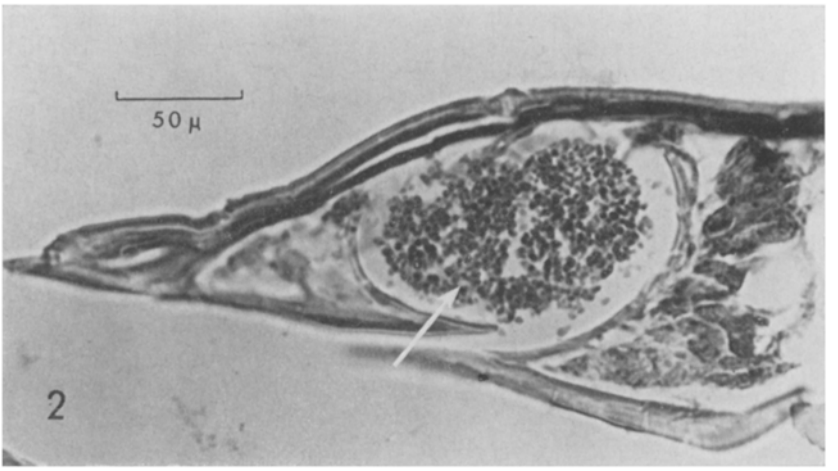

Fig. 2. Cross section of southern pine beetle mycangium with spores of SJB 133 (arrow). Section 7 μ thick, stained by Lillie's quick procedure.

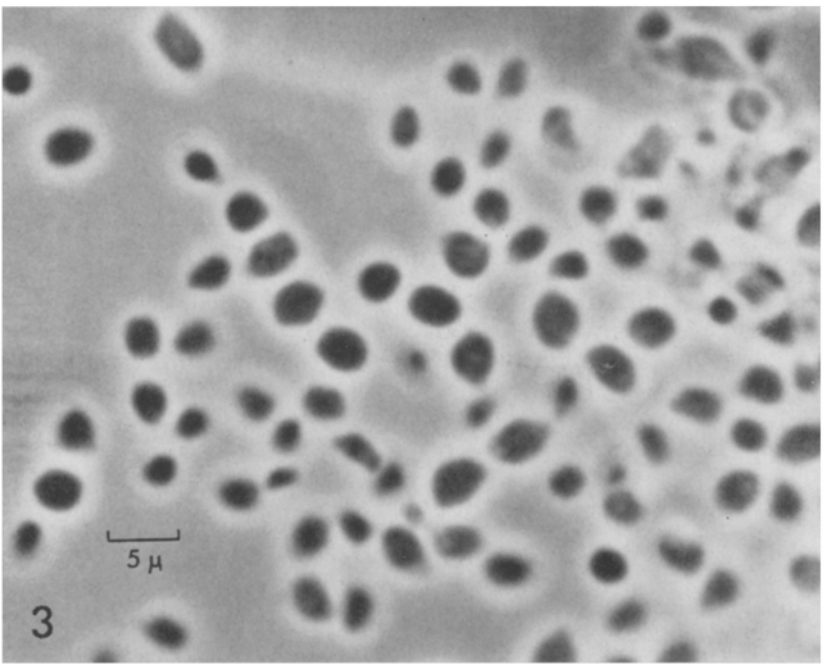

Fig. 3. Lacto-phenol mount of SJB 133 spores squeezed from mycangium.

Cells were 2.4—5.3×1.4—2.9 μ (average 3.6×2.1 μ) in size with relatively thin walls up to 0.5 μ wide. A scar was often present at the base, giving a truncated appearance to it. No conidiophores were observed in the mycangium, but the presence of secondary

conidia on short denticles indicated that reproduction occurred on sympodial extensions of conidia. On rare occasions short hyphal filaments were observed, but no conidiophores were seen on them.

Information on the incidence of SJB 133 in the mycangium and the methods of isolation may be found in a previous paper (BARRAS & PERRY, 1972). Isolates were deposited with the Centraalbureau voor Schimmelcultures (CBS 737.70).

Characteristics of SJB 133 in Pine Phloem

The following discussion is restricted to characteristics of the fungus in loblolly pine (*Pinus taeda* L.) phloem, the principal host of the southern pine beetle in Louisiana.

The fungus was often macroscopically visible as a thin, creamy-white, glistening layer lining larval galleries and the chambers of callow adults. Streak plate isolations of this layer on malt extract agar confirmed the presence of the fungus but revealed that it rarely occurred in pure culture. Isolates were also obtained from 1—2 mm cubes of phloem sampled at a lateral distance of 0.5 to 1.0 cm from galleries. Microscopic examination of phloem showed that intra- and intercellular mycelial growth was concentrated adjacent to the galleries. At locations next to active insects the fungus completely filled phloem cells lining the walls and extended into the gallery. At a lateral distance of 3 to 5 mm from the gallery, the hyphal mass thinned to a few mycelial strands per phloem cell.

The concentration of growth at the phloem surface resulted in a sporodochium-like mass of conidiophores (Fig. 4). A similar mass was also observed developing on phloem fragments produced during larval feeding on the colonized phloem. Sporogenous cells of the conidiophores were observed either as simple branches of the hyphae or as definite sympodial branches of a conidiophore. Individual sporogenous cells were 6.2—14.4 μ long by 1.2—2.9 μ wide at the base, and tapered slightly to 0.72—1.9 μ at the tip. Single thick-walled conidia were produced as terminal blown-out cells seceding at maturity (Fig. 5). Mature conidia were predominantly oval to obovate, 3.6—10.1 $\times$ 3.4—7.7 μ (avg. 7.8 $\times$ 6.2 μ) in size. Cell wall thickness varied from 1.2 to 1.5 μ, and on rare occasions a thinner transverse wall divided the conidia in half. A prominent basal or sub-basal scar was often observed.

In an effort to produce an ascigerous form, the fungus was cultured on autoclaved cross sections of loblolly pine branches. The cross sections, measuring 2 cm thick $\times$ 1.5 cm in diameter, were placed in petri dishes containing 2 % water agar and inoculated with a 3-mm cube of malt extract agar colonized with SJB 133. The fungus completely covered the disc in less than 10 days and was aerial, fluffy, and whitish with scattered synnemata rising from the surface. No sporodochial structures or perithecia were observed after one month. Mycelium width varied from less than 1 μ to 3.5 μ. The larger mycelia often produced terminal thin-walled globular

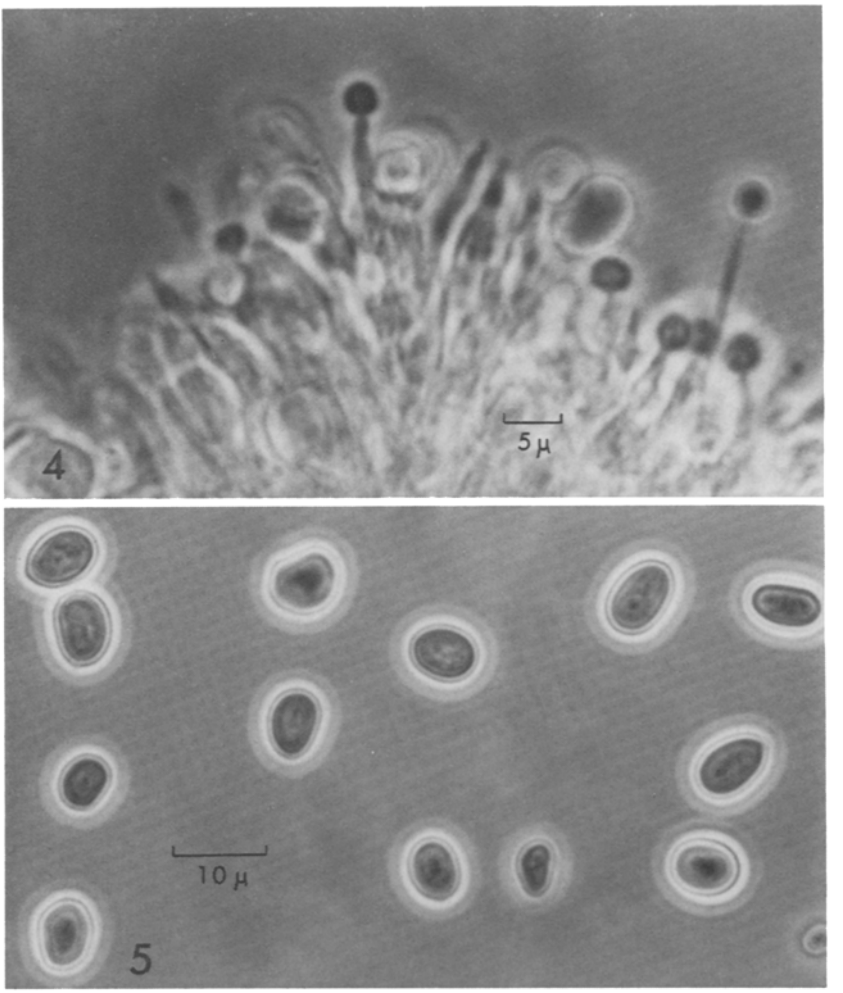

Fig. 4. Lacto-phenol mount of SJB 133 conidiophores and conidia scraped from phloem surrounding callow adult of southern pine beetle.
Fig. 5. Mature thick-walled conidia produced in larval gallery of southern pine beetle.

cells typical of monilioid ambrosia cells found in feeding chambers of xylomycetophagous scolytids and in larval galleries of the southern pine beetle.

Two types of conidia were found on pine discs: a small clavate sympodulospore (4.8×1.2 μ) and a larger thick-walled terminal spore (9.5×5.5 μ) produced on sympodial conidiophores. Basal or sub-basal scars often gave the conidia a truncated appearance.

Characteristics of SJB 133 in Cultures

Primary subcultures were established on Sabouraud glucose agar (SGA; Difco), potato-glucose agar (PGA; Difco), malt extract (Difco), glucose-blood agar (Difco brain-heart infusion agar with 5 % blood and 2 % glucose), SGA with 0.04 % cycloheximide, or potato-carrot agar (PCA) (Commonwealth Mycological Institute, p. 45, 1960). After 10—14 days at 24—27 °C colonies were flat, moist, spreading, and cream-colored. Margins were hyaline and imbedded. Discrete elevated, yeast-like or moriform moist turfs appeared irregularly throughout the colonies on most media. Superficially they resembled sporodochia, but occasionally they were sterile.

Little or no growth occurred within 5 days on media incubated at 37 °C, nor did growth resume during subsequent inoculation for 7 days at 25 °C. Pyrimidine-free medium (MARIAT *et al.*, 1962) supported a trace of growth at 24—27 °C, and none at 37 °C.

Microscopic examination revealed pronounced polymorphism in all cultures at room temperature. Thin, regularly septate, hyaline hyphae not exceeding 1.5 μ in diameter were mixed with coarse, irregularly septate hyaline hyphae 2.2—5.5 μ in diameter. The latter contained pseudohyphal segments, numerous intercalary and terminal condyloid cells, nodular bodies, and other hyphal aberrations. Sporodochial turfs consisted largely of polymorphic mycelium.

Two types of conidia were observed. Globose or subglobose conidia resembling chlamydospores were the more numerous, and arose terminally or laterally on sympodulously branched conidiophores, from which they seceded readily when mature. The conidia were 6.8—16.2 × 1.4—10.3 μ (average 11.1 × 8.5 μ), had smooth refractile walls up to 1.5 μ thick, a nucleus about 1.6 μ in diameter, and an almost inconspicuous truncate polar or subpolar basal scar not exceeding 3.0 μ in diameter. At the scar a pore through the spore wall was sometimes visible. About one-tenth of these conidia were bicellular with a plane or curved transverse septum that was usually thinner than the outer spore wall. Nuclei of didymosporous forms did not exceed 1.2 μ in diameter. Immature conidia were always amerosporous, had thinner walls than mature forms, and were smaller (Fig. 6).

Subglobose to ovate, thin-walled sympodulospores were the second, less numerous type of conidia. They were 2.0—5.5 × 1.5—5.5 μ (average 5.0 × 3.7 μ) with very narrow (0.7 μ) to very wide (2.0—2.5 μ) truncate bases. Sympodulae were either gradually tapered to *Sporothrix*-like spiculate termini, or were nearly cylindrical with roughened and somewhat geniculate termini similar to those in some *Rhinocladiella* and *Raffaelea* species. Similar conidia were also produced on short lateral spicules or pedicels on undifferentiated conidiophores (Fig. 7).

Because of the obvious polymorphism in these first cultures, an

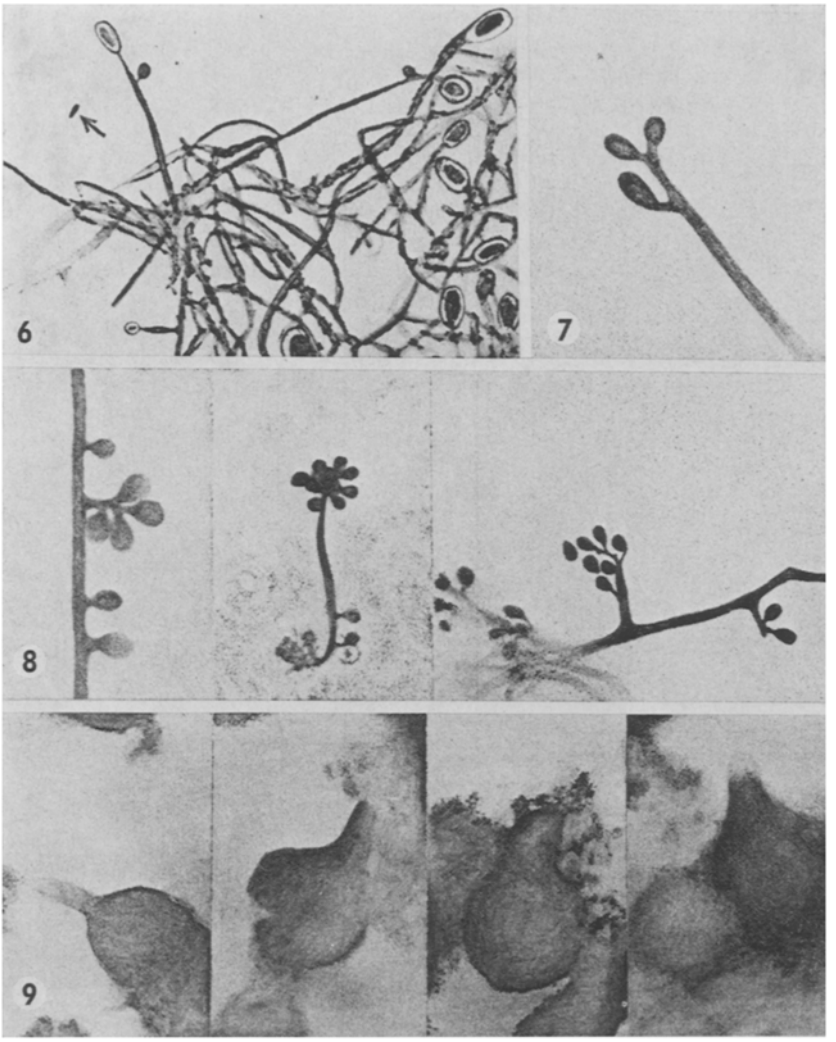

Fig. 6. Thick-walled conidia found in initial cultures of SJB **133**. One conidium (arrow) is representative of those predominating in later serial subcultures.
Fig. 7. Thin-walled, broad-based sympodulospores found in early cultures of SJB **133**.
Fig. 8. Typical *Sporothrix*-like sympodulae and conidia of SJB **133** after monomorphic stabilization by serial transfer on PCA.
Fig. 9. Mature and sclerotium-like immature perithecia imbedded in old PGA cultures of SJB **133**.

attempt was made to produce a stable monomorphic form by serial hyphal-tip transfers on PCA. After about six such transfers, colonies were flat and nearly white, with moist hyaline margins and scattered areas of dry, short aerial hyphae. Sporodochial structures

were absent. On microscopic examination regularly septate hyaline hyphae 1.1—1.6 μ in diameter were observed. Hyphal aberrations were rare, and thick-walled conidia were not seen. Sporogenous cells were simple, gradually tapered sympodulae 4—17 μ in length, distally spiculate and occasionally swollen, and either sessile or on short branches of conidiophores. Conidia were mostly obovate, 1.7—4.2 $\times$ 1.4—2.4 μ, but some were subglobose, oval, pyriform, clavate, and cuneiform. They arose both from sympodulae and from short lateral spicules on undifferentiated conidiophores. All had narrow truncate bases not exceeding 0.6 μ in diameter (Fig. 8).

Tufts of sterile synnemata, imbedded dark-brown carbonaceous sclerotium-like bodies 25—220 μ in diameter, and submerged dark-brown perithecia developed over the surfaces of the original inocula and more sparsely over remaining colony surfaces on PGA plates stored at 24—27 °C for 3 months (Fig. 9). Perithecia varied in length to a maximum of 145 μ. Bases were globose, about 40—80 μ in diameter, and lacked ornamentations other than occasional coarse, brown ventral hyphae. Necks were tapered from 15—18 μ in diameter at insertion to 10—14 μ at the ostioles. Ostiolar hyphae were numerous, hyaline, straight, and 9—14 μ long. Total length of necks, including ostiolar filaments, did not exceed 60 μ. Hyaline ascospores were roughly ellipsoid to crescent-shaped, 3.0 $\times$ 1.7—2.2 μ. Asci were not seen.

In vivo Responses to SJB 133

In its cultural characteristics SJB 133 appeared closely allied to *Ceratocystis minor* (HEDGCOCK) HUNT. Further definition of the relationship was therefore sought through virulence and serological studies. Two strains of *C. minor* (UM 5819 and 5819-A) and a monomorphic culture of SJB 133 (UM 5759) were used to prepare hyphal suspensions for intraperitoneal injection into adult CFW mice. UM 5819 is a typical form isolated from *Pinus ponderosa* LAWS. in western Montana, while UM 5819-A is a spontaneous hyaline-sectored mutant of the same strain. Details of procedures have been described elsewhere (TAYLOR, 1970).

During 20 days following injection, all animals appeared entirely normal. On the 21st day, all were sacrificed and examined for gross and microscopic evidence of infection, and for the presence of viable fungi in hepatic and splenic tissue. Results are presented in Table I. The lack of pathogenicity and longevity of SJB 133 in mice is further evidence that the fungus rapidly loses viability at 37 °C, while *C. minor* appears not only to survive, but to proliferate (TAYLOR, 1970).

Mucin-free hyphal suspensions of SJB 133 (UM 5759), *C. minor* (UM 5819 and 5819-A, and *Sporothrix schenckii* HEKTOEN & PERKINS (UM 5421) were killed by heating at 80 °C for 30 minutes, cooled, and injected into marginal ear veins of separate adult rabbits. Injections were repeated on alternate days thereafter for a total of eight injections. On the fifth and tenth days after the last

TABLE I

Fate of *C. minor* and of SJB 133 following intraperitoneal injection into mice[a,b]

	Gross pathology	Microscopic pathology	Recovery of fungi from hepatic and splenic tissue
C. minor, UM 5819	Numerous pustular lesions of liver and spleen; induration at injection site	Occasional elongate-ovate budding cells resembling *S. schenckii* in pustules and adjacent tissues	+
C. minor, UM 5819—A	Lesions as above; induration variable	As above	+
SJB 133, UM 5759	Lesions lacking; induration variable	Budding cells not seen	—

[a]) See text and TAYLOR, 1970, for details.
[b]) Accession numbers are those of the University of Montana collection.

TABLE II

Serological reactions involving SJB 133, *Ceratocystis minor*, and *Sporothrix schenckii*[a]

Antigen	Arthus	Aggln	CF
	Antiserum Directed Against *C. minor*, UM 5819		
C. minor, UM 5819	++	1:16	1:128
C. minor, UM 5819—A	++	1:16	1:32
SJB 133, UM 5759	+	1:2	1:8
S. schenckii, UM 5421	++	1:4	1:8
	Antiserum Directed Against *C. minor*, UM 5819—A		
C. minor, UM 5819	++	1:8	1:32
C. minor, UM 5819—A	++	1:8	1:256
SJB 133, UM 5759	+	1:2	1:8
S. schenckii, UM 5421	++	1:4	1:8
	Antiserum Directed Against SJB 133, UM 5759		
C. minor, UM 5819	+	1:4	1:8
C. minor, UM 5819—A	++	1:4	1:4
SJB 133, UM 5759	+	1:32	1:128
S. schenckii, UM 5421	++	1:8	1:16
	Antiserum Directed Against *S. schenckii*, UM 5421		
C. minor, UM 5819	++	1:4	1:8
C. minor, UM 5819—A	++	1:4	1:16
SJB 133, UM 5759	+	1:4	1:8
S. schenckii, UM 5421	++	1:128	1:512

[a]) Accession numbers are those of the University of Montana collection.

injection, all animals were tested for Arthus-type responses with homologous and heterologous suspensions. On the tenth day they were bled and the sera used in homologous and heterologous agglutination and complement fixation titrations (Table II).

Although it is likely that none of the reactions observed are highly specific, SJB 133 does not appear to be homologous with either *C. minor* or *S. schenckii*. However, there is evidence of some generic antigenic identity that needs further resolution.

Discussion

Interactions in Mycangium and Host Phloem

The southern pine beetle and SJB 133 have evolved an elaborate relationship characterized by the maintenance of a specialized form of the fungus in a mycangium which appears as complex as any in the strictly xylomycetophagous scolytids (ambrosia beetles) (Happ *et al.*, 1971). In addition, the fungus's ambrosial characteristics in larval galleries, its prolific production of conidia on phloem adjacent to active beetles, and its polymorphism in culture are typical qualities of mycangial fungi that provide an essential sterol for the development of at least one ambrosial beetle (Norris *et al.*, 1969; Kok *et al.*, 1970). Present evidence indicates that there is an apparently similar mutualistic symbiosis between the southern pine beetle and its mycangial fungi.

Inoculum taken from both the mycangium and insect galleries produced polymorphic forms, including two types of conidia, on various media and conifer tissue not associated with insect infestation. In contrast, monomorphic development and reproduction were observed in association with the insect and after serial passage on PCA. The monomorphic yeast-like phase induced in the mycangium is apparently controlled by chemical messengers or allomones from gland cells associated with the structure (Happ *et al.*, 1971; Barras & Perry, 1971). Ambrosial growth and mono-conidial reproduction is enhanced in the phloem during the presence of developing insects. Although little is known about this interaction, it may be controlled through a combination of another insect allomone and growth factors produced or influenced by other microbes in the phloem substrate.

Ambrosia growth is similarly induced in various stain fungi, including *Ceratocystis* spp., associated with scolytids, thus indicating parallel mutualistic adaptation (Francke-Grosmann, 1967; Graham, 1967). In addition it appears that ascigerous fungus reproduction is prevented or delayed in these associations when certain stages of the insect are present. In the southern pine beetle-fungus associations, prevention of ascigerous reproduction is significant because ascigerous *C. minor* is capable of rendering host phloem unsuitable for beetle development (Barras, 1970). Thus potential competition has been mitigated by the selection and maintenance

of SJB 133, representing a normally anascigerous variety of *C. minor* (see below). The evolution of the varietal form was probably influenced through the repeated contact with the selective chemical habitat of the mycangium. The classical ubiquitous *C. minor* transported on the adult bodies does not develop in association with larvae or callow adults, but ascocarps are widely scattered in adjacent phloem as the beetle completes development.

It is interesting to note that the mountain pine beetle (*Dendroctonus ponderosae* HOPK.) induces ambrosial growth of its associated stain fungi in pupal chambers (WHITNEY, 1971). Teneral adults fed on this growth and on phloem containing some ascocarps. As in the southern pine beetle, the 1st instar larvae were not associated with the ascigerous development of the fungi. The remaining instars fed in axenic phloem and the fungi were not again observed until the pupal stage. Although the different beetles diverge in certain aspects of their in-host habits and apparently have different relationships with the ascigerous stages of the fungi, they both ingest ambrosia growth.

Taxonomic Interpretration of Growth Studies

In its resistance to cycloheximide, production of submerged sclerotia, and general morphology of conidial and ascigerous structures, SJB 133 resembles *C. minor* as described by RUMBOLD, HUNT, and others (GRIFFIN, 1968; HUNT, 1956; RUMBOLD, 1936). It differs from *C. minor* in its diastatic activity, its entirely hyaline mycelium, its failure to survive either *in vivo* or *in vitro* at 37 °C, its antigenicity as determined by complement fixation titration, and in size and shape of its ascospores.

HEDGCOCK's original descriptions and illustrations of *C. minor* are ambiguous and do not agree well with type material (HEDGCOCK, 1906; HUNT, 1956). It is not remarkable that subsequent definition of the species became confusing, and that numerous forms which differed from type descriptions were disposed elsewhere (GRIFFIN, 1968; HUNT, 1956; WRIGHT & CAIN, 1961). The status of the taxon was clarified with HUNT's re-definition of the species and resolution of its many synonyms (HUNT, 1956). As a result, *C. minor* is now considered to be an eminently variable species, including the atypical forms described by RUMBOLD as *C. pseudotsugae* (RUMBOLD, 1936) and the unusual isolate of DAVIDSON (DAVIDSON, 1958).

Because previous interpretations of *C. minor* have been inclusive rather than exclusive, and because mutative variants from the type have been noted previously (see TAYLOR, 1970), a new binomial for SJB 133 may not be warranted at this time.

However, we believe that the morphological, physiological, and antigenic differences which we have observed among SJB 133 and strains of *C. minor* represent significant genotypic differences as

well. We propose, therefore, that SJB 133 warrants varietal rank in *Ceratocystis minor* (HEDGCOCK) HUNT.

Ceratocystis minor (HEDGCOCK) HUNT var. *barrasii* J. TAYLOR var. nov.

Hyphis in cultura semper albis vel lacteis. Perithiciis in cultura immersis, atro-brunneis vel nigris, globosis, 40—80 μ diam.; collo brunneo, 30—60 μ longo, saepe curvo, ad basem 15—18 μ ad apicem 10—14 μ diam.; hyphis ostioli hyalinis, rectis, 9—14 μ longis; ascis evanescentibus, non visis; ascosporis hyalinis a fronte conspectis ellipsoideis, a latere conspectis cum figura segmenti pomi citri, sine vagina, 3.0×1.7—2.2 μ; conidiis numerosis, unicellulis, hyalinis, plerumque obovatis, 1.7—4.2×1.4—2.4 μ, in conidiophoris generis *Sporothricis* formatibus.

Summary

A symbiotic fungus (SJB 133) of the southern pine beetle, *Dendroctonus frontalis* ZIMM., was observed in the prothoracic mycangium as small (3.6×2.1 μ), thin-walled spores that produced sympodulate secondary conidia. In conifer phloem, the fungus developed an often macroscopically visible sporodochium-like layer of sympodial conidiophores and large thick-walled conidia (7.8×6.2 μ) lining the galleries of developing beetles. Primary subcultures on various media were polymorphic and produced two types of conidia representing a *Sporothrix* imperfect state of *Ceratocystis*. The largest (avg. 11.1×8.5 μ), and most numerous, arose terminally or laterally on sympodulously branched conidiophores and the smallest were thin-walled sympodulospores (avg. 5.0×3.7 μ). A monomorphic form was observed after six transfers on potato-carrot agar. Perithecia and hyaline, roughly ellipsoid to crescent-shaped ascospores (3.0×1.7—2.2 μ) were observed on potato-glucose agar after three months. A further definition of the fungus was obtained by comparing *in vivo* and immunological reactions among SJB 133, two strains of *Ceratocystis minor* (HEDGCOCK) HUNT, and *Sporothrix schenckii* HEKTON & PERKINS. These studies revealed that SJB 133 resembled *C. minor* in resistance to cycloheximide, production of submerged sclerotia, and morphology of conidial and ascigerous structures. It differed in its diastatic activity, entirely hyaline mycelium, failure to survive either *in vivo* or *in vitro* at 37 °C, its antigenicity, and in size and shape of ascospores. The fungus was described as *Ceratocystis minor* (HEDGCOCK) HUNT var. *barrasii* J. TAYLOR var. nov.

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