Light- and electron-microscopic observations of *Cladosporium* sp. growing on basidia of *Exobasidum camelliae* var. *gracilis*

C.W. Mims, R.T. Hanlin, and E.A. Richardson

Abstract: Basidia of the plant-pathogenic fungus *Exobasidium camelliae* var. *gracilis* Shirai became exposed on the abaxial side of an infected leaf of *Camellia sasanqua* Thunb. following the sloughing of the undersurface of the leaf. Basidia were formed in tremendous numbers in a distinct hymenium that appeared as a white, felt-like layer. Subsequently, colonies of another fungus, *Cladosporium* sp. appeared, initially as tiny dark dots on this white layer, but quickly increased in size to form larger circular colonies that were olive-brown to dark brown in color. Adjacent colonies sometimes merged to form larger growths with irregular margins that often covered much of the hymenium on the underside of an *E. camelliae*-infected leaf. The hyphae that made up a young *Cladosporium* sp. colony were primarily confined to the surface of the *E. camelliae* hymenium, and we found no evidence that hyphae actually penetrated living basidia. However, *E. camelliae* basidia overrun by *Cladosporium* sp. eventually showed signs of necrosis and finally collapsed and died, creating a slightly sunken area in the hymenium. Hyphae of *Cladosporium* sp. grew throughout the remains of the dead basidia, but did not appear to spread into the leaf tissue above the pseudoparenchymatous layer of hyphae that gave rise to the basidia. Based upon our observations, it is clear that *Cladosporium* sp. is a necrotrophic mycoparasite. More specifically, it qualifies as a contact necrotrophic, since it kills basidia without first penetrating them with its hyphae.

Key words: mycoparasitism, fungicolous, necrotrophic parasite.

Résumé : Les basides du champignon phytopathogène *Exobasidium camelliae* var. *gracilis* Shirai, apparaissent du côté abaxial des feuilles infectées du *Camellia sasanqua* Thunb., suite au décollement de la surface inférieure de la feuille. Les basides se forment en très grand nombre sur un hyménium distinct, qui apparaît sous forme d'une couche blanche feutrée. Les colonies du *Cladosporium* sp. apparaissent d'abord comme de petits points, sur cette couche blanche, mais augmentent rapidement de dimension pour former de larges colonies circulaires, allant du brun olivâtre au brun foncé. Des colonies adjacentes se fusionnent occasionnellement les unes aux autres pour former des colonies plus larges aux pourtours irréguliers, lesquelles recouvrent souvent une bonne partie de l'hyménium, sur la face inférieure des feuilles infectées par l'*E. camelliae*. Les hyphes constituant une jeune colonie sont surtout confinées à la surface de l'hyménium, mais on observe aucune preuve que les hyphes du *Cladosporium* sp. pénètrent effectivement les basides vivantes. Cependant, les basides recouvertes de *Cladosporium* sp., montrent éventuellement des signes de nécrose et s'affaissent avant de mourir, créant ainsi une faible dépression dans l'hyménium. Les hyphes du *Cladosporium* sp. poussent parmi les résidus de basides mortes, mais ne semblent pas s'étendre dans les tissus foliaires au-delà de la couche parenchymateuse des hyphes qui donnent naissance aux basides. Sur la base de ces observations, il est clair que le *Cladosporium* sp. est un parasite nécrotrophe. Plus spécifiquement, on doit le considérer comme un nécrotrophe de contact, puisse qu'il tue les basides sans d'abord les pénétrer avec ses hyphes.

Mots-clés : mycoparasitisme, fongicole, parasite nécrotrophe.

[Traduit par la Rédaction]

Introduction

Cladosporium is a very large anamorphic fungal genus containing saprobic, endophytic, plant-pathogenic, and fungicolous taxa (Heuchert et al. 2005). Fungicolous species are defined herein as those that obtain their nutrients from other fungi, although some workers have also used the term to describe fungi that consistently grow on lichens. There is extensive literature on fungicolous fungi (see Gams et al. 2004), although it is somewhat confused, as the term fungicolous has been used to describe saprobes, commensals, and parasites. In the case of the genus *Cladosporium*, Heuchert et al. (2005) have identified 26 species that appear to grow exclusively on other fungi. Although these species have typically been referred to as mycoparasities, very little is known about the exact nature of the trophic relationships that exist between most of these species and the fungal structures they utilize as substrates. At this point, mycoparasitic fungi have been placed in one of two large categories, depending upon whether the hyphae or cells of their hosts are killed (Jeffries 1995; Gams et al. 2004). The first category, the so-called biotrophs, establish what have been de-

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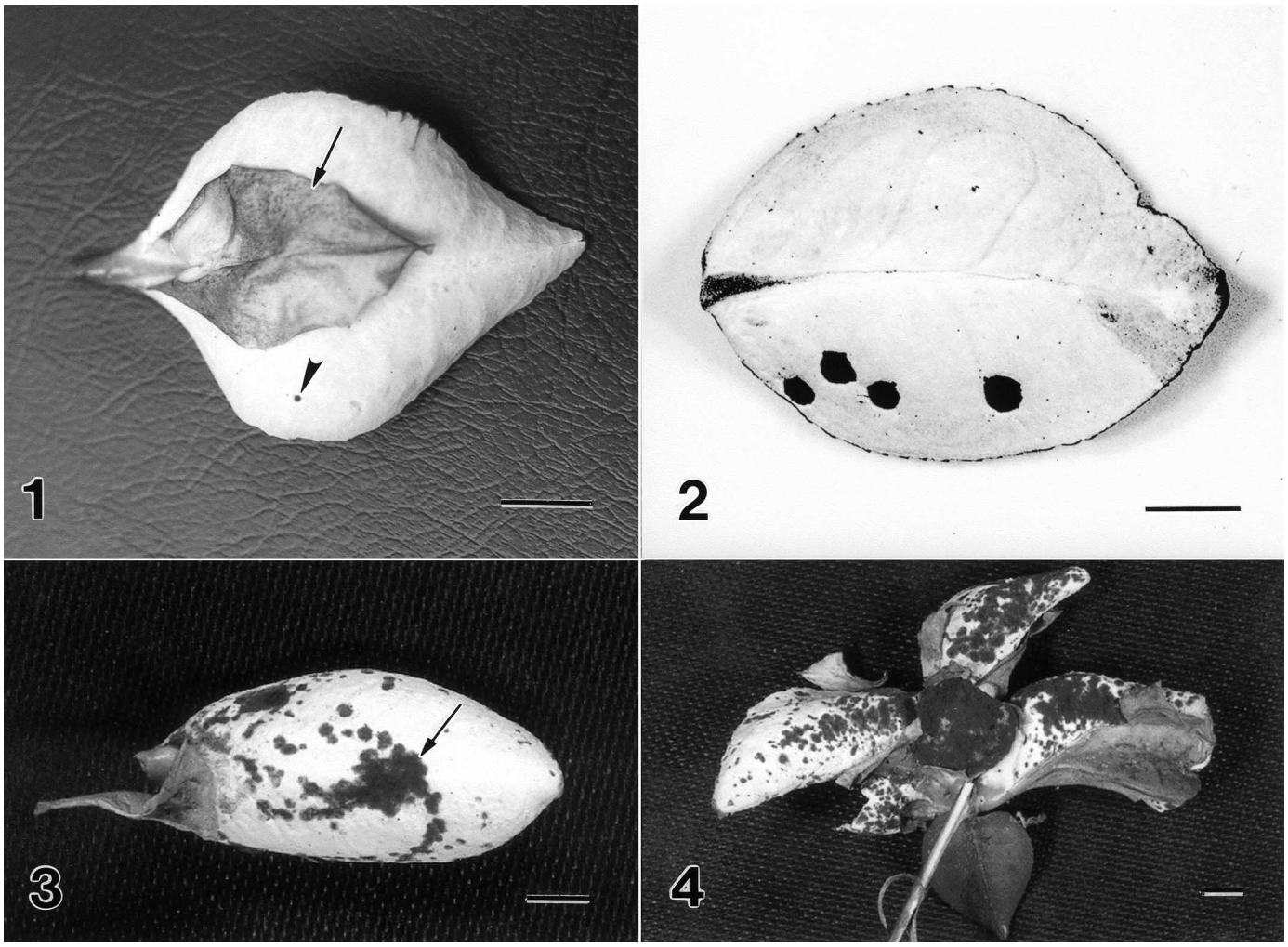
Figs. 1–4. Views of the abaxial surfaces of *Camellia sasanqua* leaves infected by *Exobasidium camelliae* var. *gracilis*. Fig. 1. Leaf with its detached and withered underside (arrow) still covering a portion of the white hymenium of *E. camelliae*. A single tiny *Cladosporium* sp. colony is visible (arrowhead). Fig. 2. Four large *Cladosporium* sp. colonies growing on an exposed hymenium. Fig. 3. A leaf with numerous *Cladosporium* sp. colonies, some of which have fused to form a large growth with an irregular margin (arrow). Fig. 4. Numerous leaves from a single shoot. The hymenium on each leaf is almost completely overrun by *Cladosporium* sp. colonies. Scale bars = 1 cm.



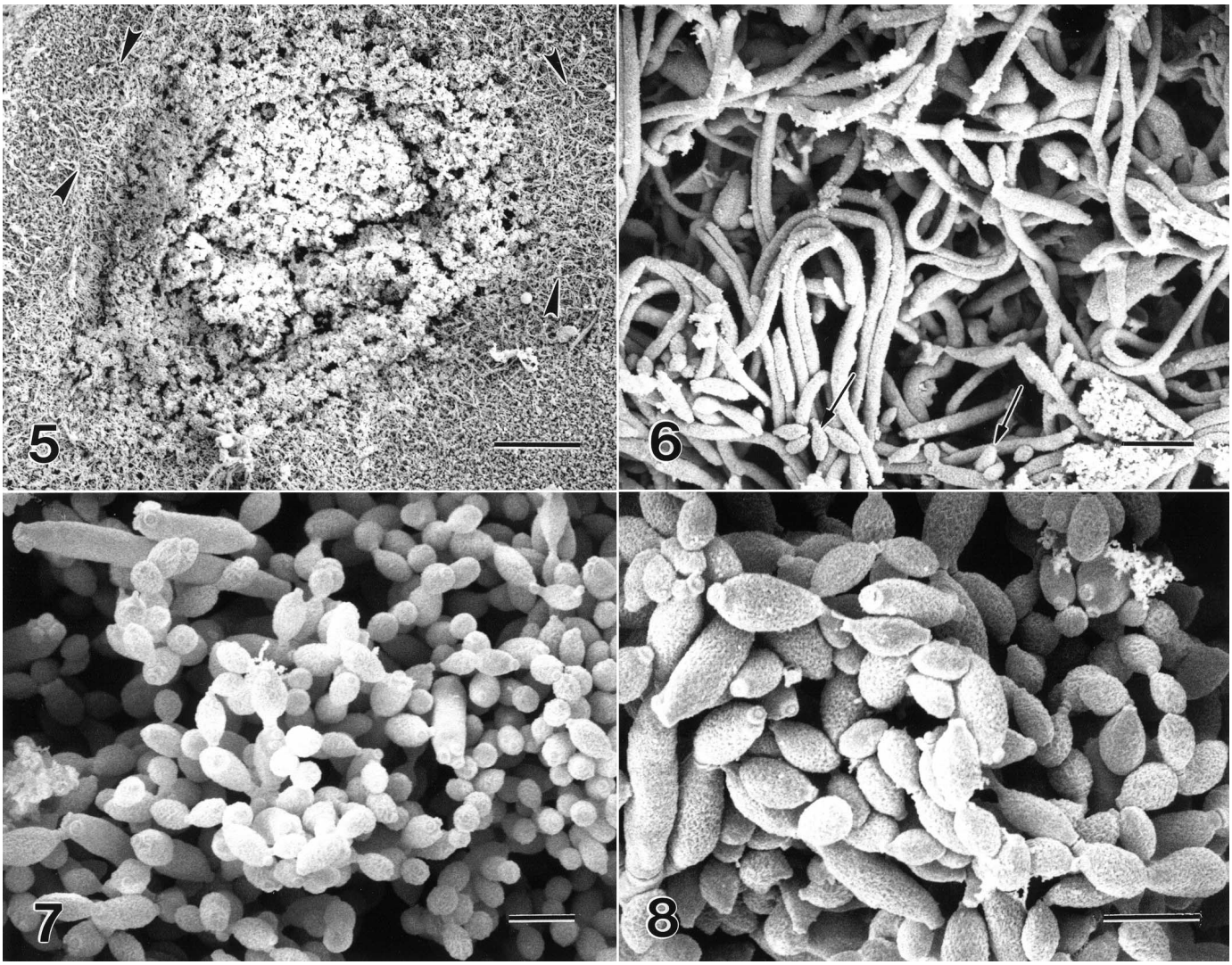
scribed as "balanced relationships" (Hawksworth et al. 1995) with their hosts, in which host hyphae or cells remain alive. The second category, the necrotrophs, kill host structures. Necrotrophic mycoparasites can be further divided into two groups: (*i*) contact forms that do not penetrate their hosts, and (*ii*) invasive forms that penetrate their hosts (Jeffries 1995; Gams et al. 2004).

In this study we used a combination of light (LM) and electron microscopy in an attempt to determine whether an apparently undescribed species of *Cladosporium* found growing on basidia of the plant-pathogenic fungus *Exobasidium camelliae* var. *gracilis* Shirai (hereinafter *E. camelliae*), is a biotrophic or a necrotrophic mycoparasite. The basidia of *E. camelliae* arise from a pseudoparenchymatous layer of hyphae that develops in the intercellular spaces of an infected leaf of *Camellia sasanqua* Thunb. 4–6 cell layers above the lower epidermis, and become exposed to the environment following the sloughing of the lower surface of an infected leaf (Wolf and Wolf 1952).

Materials and methods

The samples examined in this study came from leaves of *C. sasanqua* naturally infected by *E. camelliae*. Infected leaves bearing exposed basidia of *E. camelliae* overrun by colonies of *Cladosporium* sp. were collected in the field in April 2005 and prepared for study with both LM and electron microscopy according to the following procedures: A razor blade was used to remove small pieces of those infected leaves whose undersurfaces were covered with *E. camelliae* basidia infected with *Cladosporium* sp.; samples for scanning electron microscopy (SEM) were prepared using a standard fixation procedure (Enkerli et al. 1997) involving the use of glutaraldehyde and OsO_4. Following fixation, samples were washed in distilled water, dehydrated in a graded ethanol series, and critical-point dried. Samples were mounted on specimen stubs using conductive tape, sputter-coated with gold, and examined using a JEOL 6400 microscope operating at 15 kV. For transmission electron

Figs. 5–8. Scanning electron micrographs of *Cladosporium* sp. growing on the basidia of *E. camelliae*. Fig. 5. A small *Cladosporium* sp. colony with hyphae (arrowheads) spreading from the margin onto the hymenium of *E. camelliae*. Scale bar = 250 μm. Fig. 6. Slightly higher magnification of the margin of a colony showing numerous hyphae and a few conidia (arrows). Scale bar = 10 μm. Fig. 7. View of the center of a colony showing a mass of conidiogenous cells and conidia. Scale bar = 5 μm. Fig. 8. Slightly higher magnification of conidiogenous cells and conidia. Scale bar = 5 μm.

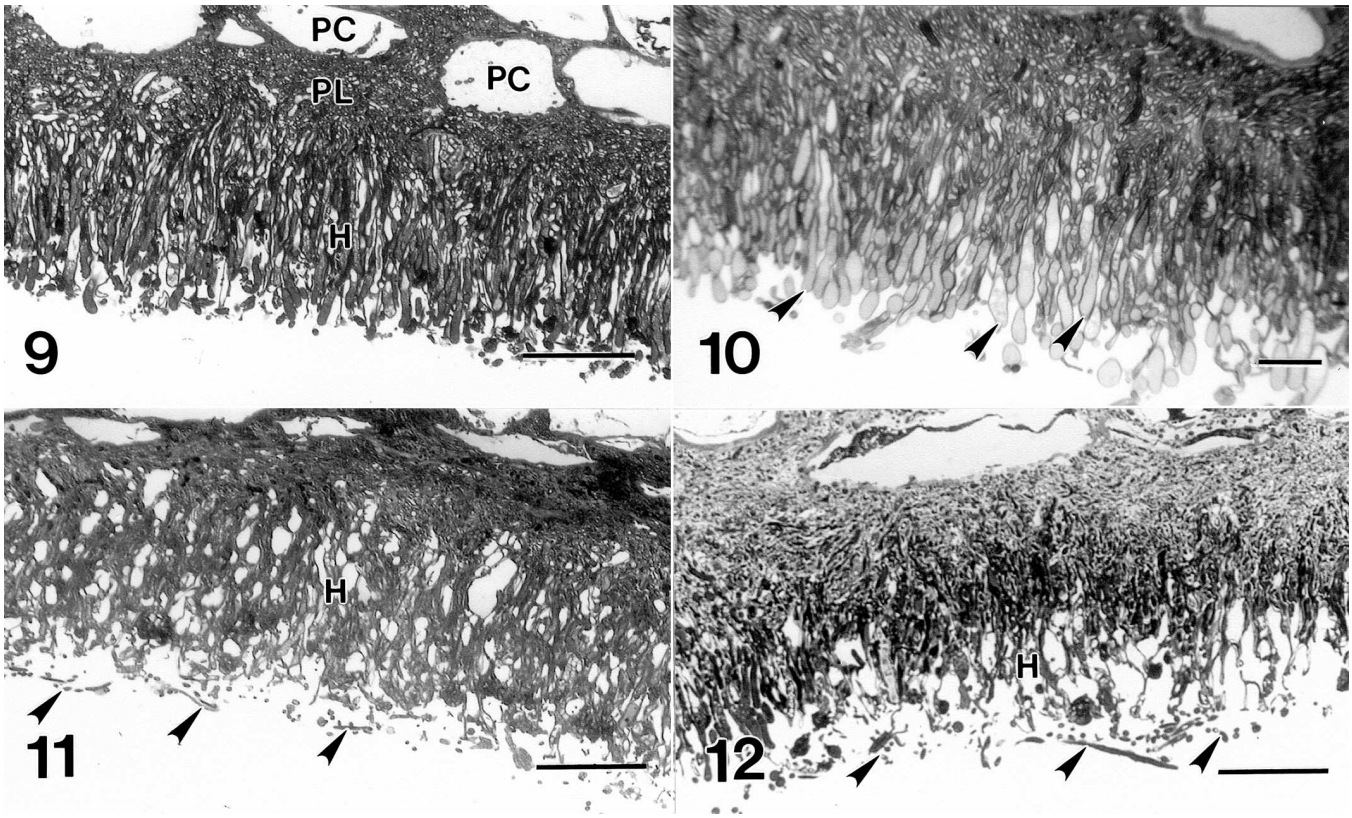


microscopy (TEM), samples were fixed as previously described, dehydrated, and infiltrated with Spurr's resin (Taylor and Mims 1991); following resin polymerization, thin sections of the samples were cut using an ultramicrotome equipped with a diamond knife, collected on slot grids, allowed to dry on formvar-coated aluminum racks (Rowley and Moran 1975), post-stained with uranyl acetate and lead citrate, and examined with a Zeiss 902A microscope operating at 80 kV. For LM, ca. 1 μm thick sections of resin-embedded samples were cut with a diamond histology knife, collected on glass microscope slides, stained with toluidine blue O and examined and photographed using bright-field LM.

Results

Leaves of *C. sasanqua* infected by *E. camelliae* first manifested in early April in the Athens, Georgia, area. Although the overall shape of an infected leaf was similar to that of a healthy leaf, infected leaves were much larger and thicker than healthy leaves, and were light green compared to the dark green of healthy leaves. Developing basidia of *E. camelliae* formed in a definite hymenium that became exposed following the sloughing of the abaxial surface of an infected leaf (Fig. 1). Basidia were formed in tremendous numbers in a distinct hymenium that appeared as a white, felt-like layer on the leaf surface. Although we rarely observed colonies of *Cladosporium* sp. on *E. camelliae* basidia in early April, they became extremely common later in the month as average daily temperatures increased. Colonies of *Cladosporium* sp. first appeared as tiny dark circular spots that developed about a week after the host basidia became exposed (Fig. 1). These tiny spots quickly increased in size to form larger, slightly sunken colonies (Fig. 2) that were olive-brown to dark brown in color. Adjacent colonies often merged to form large growths with irregular margins (Fig. 3). By the end of April, *Cladosporium* sp. colonies

Figs. 9–12. Light micrographs of thick sections of resin-embedded samples stained with toluidine blue O. Fig. 9. A healthy hymenium (H) of *E. camelliae*. Note the pseudoparenchymatous layer of hyphae (PL) that separates the hymenium from the plant leaf cells (PC) above. Scale bar = 80 μm. Fig. 10. Higher magnification view of a number of healthy developing basidia (arrowheads). Scale bar = 10 μm. Figs. 11. and 12. Examples of basidia overrun and killed by hyphae of *Cladosporium* sp. The hymenium is shown (H), but few, if any, intact basidia are evident. Hyphae and conidia of *Cladosporium* sp. are indicated the arrows. Scale bars = 80 μm.



could be found on virtually every *E. camelliae*-infected leaf and, in many cases, almost completely overran the hymenium on an infected leaf (Fig. 4).

SEM was useful for showing *Cladosporium* sp. growing on the basidia of *E. camelliae*. Figure 5 shows a relatively small colony and hyphae can be seen around the margin, spreading out over the surface of the hymenium. Numerous hyphae and a few conidia from near the margin of a colony are shown at higher magnification in Fig. 6. Micrographs taken near the slightly sunken central portion of a well-developed colony revealed huge masses of conidia and conidiogenous cells (Figs. 7 and 8). Because these structures obscured the associated basidia, LM and TEM proved to be much more valuable than SEM for examining details of the interactions of *Cladosporium* sp. hyphae with basidia.

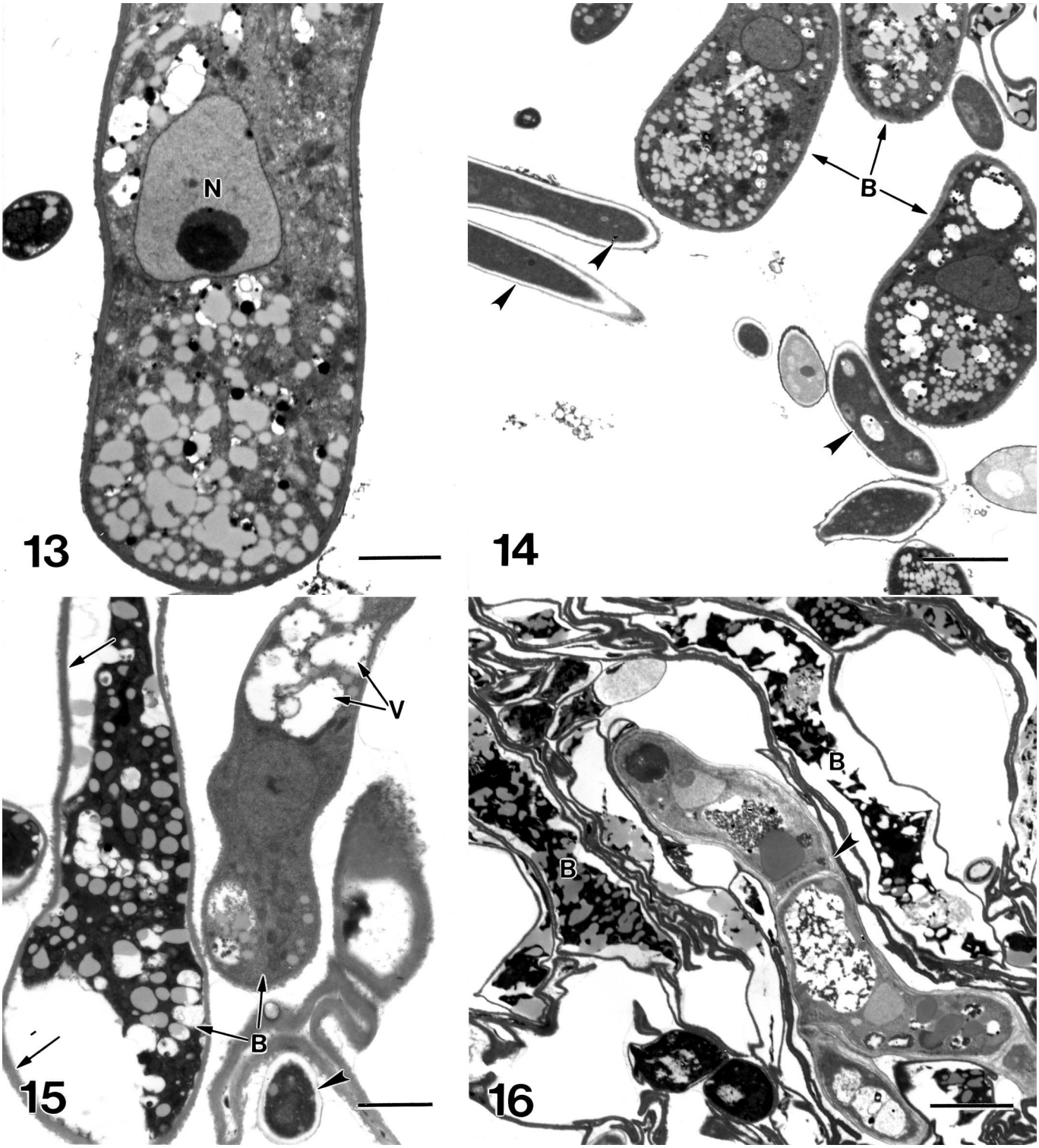
Figures 9 and 10 are light micrographs showing sections of healthy *E. camelliae* basidia. As evident in Fig. 9, basidia were produced in a distinct hymenium separated from the overlying host-leaf cells above by a pseudoparenchymatous layer of *E. camelliae* hyphae. At higher magnification the developing basidia appeared as swollen hyphal tips (Fig. 10). In contrast, Figs. 11 and 12 show basidia overrun by *Cladosporium* sp. colonies. Virtually all of these basidia appear collapsed and dead. Based upon our observations, few if any developing basidia overrun by *Cladosporium* sp. appeared to live long enough to form basidiospores. Although *Cladosporium* sp. hyphae were readily apparent on the hymenium surface (Figs. 11 and 12) with LM, it was very difficult to identify hyphae between the dead basidia.

Ultrastructural observations revealed that each healthy developing *E. camelliae* basidium was uninucleate and packed with dense cytoplasm. Small vacuoles were shown to contain electron-dense deposits (Figs. 13 and 14.). On a hymenium infected with *Cladosporium* sp. the basidia around the margin of a colony also appeared healthy when first overrun by hyphae (Fig. 14). These hyphae initially appeared to be confined to the surface of the hymenium (Fig. 14) and we saw no evidence that intact basidia were penetrated by hyphae. However, evidence of necrosis soon became apparent within basidia overrun by *Cladosporium* sp. hyphae (Fig. 15). The earliest evidence of necrosis we observed was the formation of large vacuoles in the basidium cytoplasm (Fig. 15). The contents of dying basidia became more electron-dense and the cytoplasm pulled away from the basidium wall (Fig. 15). Dead basidia eventually collapsed and disintegrated (Fig. 16). With TEM it was possible to identify *Cladosporium* sp. hyphae growing throughout the remains of dead basidia (Fig. 16). However, these hyphae did not appear to grow through the pseudoparenchymatous layer of *E. camelliae* hyphae and into the overlying leaf tissue.

Discussion

Our observations clearly indicate that *Cladosporium* sp. is

Figs. 13–16. Transmission electron micrographs of basidia of *E. camelliae* and hyphae of *Cladosporium* sp. Fig. 13. A healthy developing basidium. A single nucleus (N) is visible. Scale bar = 1.5 μm. Fig. 14. *Cladosporium* sp. hyphae (arrowheads) associated with three intact basidia (B) near the margin of a colony. Scale bar = 2.5 μm. Fig. 15. Hypha of *Cladosporium* sp. (arrowhead) near two necrotic basidia (B). Note the vacuoles (V) in one of the basidia. The cytoplasm of the other basidium has pulled away from the basidium wall. Scale bar = 1.5 μm. Fig. 16. *Cladosporium* sp. hypha (arrowhead) growing through the collapsed remains of dead *E. camelliae* basidia (B). Scale bar = 2.5 μm.



parasitic on the basidia of *E. camelliae*. Virtually all of the basidia overrun by a colony of *Cladosporium* sp. were killed prior to forming basidiospores. Since living basidia did not appear to be penetrated by the hyphae of *Cladosporium* sp., this mycoparasite should be considered a contact necrotroph rather than an invasive necrotroph that enters living cells of its host. It appears that basidia died as the result of exposure to one or more toxic compounds and (or) enzymes released by hyphae of *Cladosporium* sp. A review of the literature indicates that various other fungicolous species of the genus *Cladosporium* are known to produce toxic compounds as well as enzymes that either inhibit spore germination in their host fungi or are involved in the penetration of host spores and germ tubes. To date, most of these data have come from the study of *Cladosporium tenuissimum* Cooke, a species known to parasitize a number of different rusts including *Melampsora larici-populina* Kleb. (Sharma and Heather 1987, 1988), *Cronartium flaccidum* (Alb. et Schwein.) G. Winter (Moricca et al. 2001), *Peridermium pini* (Pers.) Lév. (Moricca et al. 2001), and *Uromyces appendiculatus* (Pers.:-Pers.) Unger (Assante et al. 2004). *Cladosporium tenuissimum* is also necrotrophic, but unlike the species of *Cladosporium* studied here, it is an invasive necrotroph that enters the living host spores and germ tubes before killing them (Assante et al. 2004). In the case of *C. flaccidum* and *P. pini*, aeciospores are attacked by *C. tenuissimum*, while in *M. larici-populina* and *U. appendiculatus*, it is the urediniospores that are parasitized. Ungerminated conidia of *C. tenuissimum* attached to the surfaces of urediniospores of *U. appendiculatus* have also been shown to reduce the rate of spore germination (Sharma and Heather 1981, 1988; Assante et al. 2004). Likewise, crude extracts from conidia inhibit host-spore germination and also reduce the radial growth of the hyphae of several plant-pathogenic fungi (Sakagami et al. 1995;). Information on some of the compounds identified in these extracts can be found in Moricca et al. (2001) and Nasini et al. (2004).

In addition to *C. tenuissimum* mentioned above, a number of other species of *Cladosporium* also attack various rust fungi (Tsuneda and Hiratsuka 1979; Sharma and Heather 1980; Srivastava et al. 1985; Uma and Taylor 1987; Morgan-Jones and McKemy 1990; de Nooij and Paul 1992; Barros et al. 1999). While some of these are clearly invasive necrotrophs, information on the trophic modes of other species is not available. Still other *Cladosporium* species have been reported to parasitize other types of pathogenic plant fungi, including downy and powdery mildews, *Monilinia laxa*, and some species of *Taphrina* (Heuchert et al. 2005). Unfortunately, detailed information regarding the trophic relationships that exist between these latter *Cladosporium* species and their non-rust hosts also is lacking.

In closing we should note that a species of *Cladosporium* currently known as *Cladosporium exobasidii* Jaap. has been reported on *Exobasidium* sp.-incited galls produced on various host plants in Europe. Previously known as *C. fuligeneum*, this fungus has been found on galls produced on certain members of the Ericaceae infected by *Exobasidium rhododendri* Cram. and *Exobasidiom vaccinii* (Fuckel) Wor. (Heuchert et al. 2005). A recent description of *C. exobasidii* has been provided by Braun (2001) who described the fungus as a hyperparasite. However, to our knowledge, precise information relating to the physical and nutritional relationships between *C. exobasidii* and the galls on which it grows is not available. Although we have not been able to compare the isolate of *Cladosporium* sp. examined in this study with actual samples of *C. exobasidii*, at this point we do not believe that the two organisms are conspecific. We hope that molecular studies currently underway will elucidate the exact relationship between these two organisms.

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