Association of *Botrytis cinerea* with Grape Berry Moth Larvae

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ABSTRACT

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The potential role of the larvae of *Lobesia botrana*, the grape berry moth, as a dispersal agent for *Botrytis cinerea* has been studied with scanning electron microscopy. Numerous conidia were trapped, mainly in the ornamentations of segments cuticle. Dispersal of the pathogen via the larval feces was also demonstrated to be possible. Ingested pieces of conidiophores and conidia observed in the digestive tract remained typically shaped. The germination ability of conidia extracted from the gut was not modified, and *B. cinerea* remained viable inside feces. In vineyards near Bordeaux, *B. cinerea* was isolated from 95 and 35% of the second generation larvae in 1987 and 1988, respectively. The factors that may be involved in the infection process are discussed.

Additional keywords: epidemiology, grey mold, tortricidae, vection, vine.

Botrytis cinerea Persoon is a destructive pathogen of a wide range of plants throughout the world. In grapes (*Vitis vinifera*) substantial losses due to this fungus usually occur near the period of harvest. McClellan and Hewitt (13) distinguished this common rot from the early Botrytis rot, which appears near the period of véraison (6), the stage when grape berries beginning to ripen lose their green color. The consequences of the disease are of great importance to the quality of wines (16).

Several authors noted that grapes heavily infested with the grape berry moth *Lobesia botrana* (Denis & Schiffermueller) (Lepidoptera: Tortricidae) are often severely attacked by *B. cinerea* (3,7,18,21). This moth is one of the most destructive insects in the southern European vineyards, where it generally completes three generations a year. The damage on the surfaces of green or ripe berries caused by the larvae of the last two generations is often the most serious because of the risk of Botrytis rot epidemics.

A similar phenomenon has been reported with the lepidopterans *Argyrotaenia pulchellana* Haw. and *Eupoecilia ambiguella* Hb. (22,15). Agrios (1) suggested that these larvae bore openings through which windborne conidia of *B. cinerea* enter the grapes and cause them to rot, but no experimental work related to this association has been published.

In strawberries, bees can disperse the conidia of *B. cinerea* among flowers (11). In broad beans, effects of insect activity on the epidemiology of the rot due to *Botrytis fabae* Sard. are better known. Last (12) observed an increase in infectivity of conidial suspensions added with the honeydew of *Aphis fabae* Scop. and attributed this effect to the sucrose and glucose of the honeydew. Microscopic examination of the bodies of thrips and isolations of fungi showed that *B. fabae* can be spread by thrips (14). Blakeman (2) expressed the view that the insect activities probably influence plant pathogenic fungi by providing sites for entry, by distributing spores of pathogens, and by increasing the supply of nutrients on aerial plant surfaces.

The objective of the present work is to study the relationship between the grape berry moth larvae and *B. cinerea*. It was first necessary to demonstrate that the pathogenic fungus was in or on the larvae to provide data showing that the transport of conidia of *B. cinerea* by these larvae was possible.

MATERIALS AND METHODS

B. cinerea. A virulent isolate (R1) of *B. cinerea* was used throughout the study. This isolate was obtained originally in 1982, from the cultivar Sauvignon in a vineyard near Bordeaux. Stock cultures were maintained on 1.5% malt agar. To maintain virulence, once a year spores from stock cultures were inoculated onto rooted cuttings of grape vines in the greenhouse. The fungus was then reisolated and cultured again on malt agar. For use in this study, well sporulating cultures were obtained after a 2-wk incubation at 12:12 (L:D) photoperiod and at 20 C.

Vineyard insects. In 1987 and 1988, insects of the second generation were obtained in early August on the grape cultivar Sauvignon in the INRA experimental vineyard near Bordeaux. Larvae were captured from clusters shaken over a sterile petri dish. Larval feces were also collected from the same clusters. To determine if the larvae were contaminated by *B. cinerea*, they were then plated on 1.5% malt agar with 250 mg/L of chloramphenicol. *B. cinerea* was identified with the aid of a stereomicroscope after 11 days of incubation at 20 C. Four and six replications each of 10 larvae were studied in 1987 and 1988, respectively.

Laboratory-grown insects. The larvae completed their growth on a semi-synthetic diet in the laboratory (J. Stockel, *personal communication*). They were maintained in an air-conditioned room, at 16:8 (L:D) photoperiod, 20 C, and 70% RH. Larvae were brought into contact with the fungus on the surface of petri dishes containing sporulating *B. cinerea*.

Scanning electron microscopy (SEM). Laboratory-grown larvae in their fourth instar were brought into contact with *B. cinerea* for 1 hr, and larvae with their feces were collected in the vineyard. The samples were fixed for 12 hr in a solution of 6.25% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4. They were then rinsed several times with fresh buffer, dehydrated through graded ethanols, and CO_2 critical point dried. They were mounted on aluminum stubs, coated with carbon by vaporization on a rotating stage and then with a gold-palladium alloy by sputtering. The observations were made with an ETEC Autoscan scanning electron microscope operated at 5, 10, or 20 kV.

Transmission electron microscopy (TEM). The samples were fixed for 2 hr in the same fixative washed in the same buffer and postfixed for 1 hr in 1% osmium tetroxide in cacodylate before dehydrating through graded ethanols, through propylene oxide and were then embedded in Epon 812. The ultrathin sections were contrasted by uranyl acetate and lead citrate. Polysaccharids of the peritrophic membrane were identified and contrasted using the PATAg reaction (20). The observations were made with a JEM 1200 EX transmission electron microscope.

Light microscopic observations of *B. cinerea* in the gut. Preliminary observations on the ingestion of *B. cinerea* by the

larvae were made with an Olympus BH-2 light microscope. Larvae in their fifth instar were fed on *B. cinerea* for periods of 15, 30, 60, or 120 min. The midgut was removed and stained with 0.1% cotton blue in lactic acid. For each group, 40 midguts were examined under the microscope with a micrometer grid. The length of the midgut portion including conidia or conidiophores was measured and related to the midgut total length. Resulting percentages were transformed with the arcsin transformation to stabilize variances. The means were compared at $P = 0.05$ using Newman and Keuls' test (19).

Isolation of viable *B. cinerea* from larval feces. Experiments were made to determine whether viable *B. cinerea* was present inside the feces. Laboratory-grown larvae in their fifth instar were fed for 24 hr in petri dishes containing sporulating *B. cinerea*. The feces excreted during the following 5 hr were collected. To avoid external contamination by *B. cinerea*, 160 samples of these feces were individually surface-sterilized in a 50-g/L solution of calcium hypochlorite (70% active Cl) for 1 min, then rinsed several times in sterile distilled water. These feces were incubated on 1.5% malt agar for 1 wk at 20 C to isolate *B. cinerea*.

Feces from laboratory-grown larvae that were not fed on *B. cinerea* served as the control and were treated in three different ways as follows: Ninety-five were directly plated and incubated as previously described. Seventy-five were brought into contact with *B. cinerea* for 5 hr, then plated. One hundred ninety-five were brought into contact with *B. cinerea* for 5 hr, then surface-sterilized and plated.

Germination ability of conidia isolated from parts of the gut. Laboratory-grown larvae in their fifth instar were fed on *B. cinerea* for at least 24 hr. They were then examined against the light to differentiate the larvae with guts darkened by the presence of spores and conidiophore pieces. Forty of these larvae were killed with ethyl-acetate vapors. After having been rinsed three times with sterile distilled water, they were dissected in a sterile Ringer saline ($CaCl_2$, 0.021 g; KCl, 0.035 g; NaCl, 0.75 g in 100 ml of sterile distilled water). The stomodaeum and the proctodaeum were removed with sterile forceps. They were then individually crushed in 150 μl of sterile distilled water to release the internal spores. After an 18-hr incubation on 1% water agar at 20 C, the germination rate was assessed by examining four replications of 100 conidia. A conidial suspension from the culture on which each larva was fed was used as control. The mean germination rates were compared at $P = 0.05$ using a paired Student's *t*-test (19).

RESULTS

Aspects and distribution of conidia on larval cuticle. *Laboratory study.* Numerous conidia could be seen by SEM on all parts of the cuticle of laboratory-grown larvae brought into contact with sporulating *B. cinerea* for 1 hr (Fig. 1). The conidia were ellipse-shaped, 6 μm in diameter and 10 μm in length, with a typical prominent abscission scar. Their uneven surface was sometimes covered with small warts (Fig. 2). The head-capsule, composed of several smooth sclerites, tended to trap conidia in the low-lying areas. Most of the conidia were found aggregated around the stemmatae and at the base of the setae. In addition, a few conidia were found near the mouthparts (Fig. 3). On the larval segments three main types of ornamentation could be distinguished concerning differences in conidia trapping. First, the sclerotized areas were sculptured in fine ridges (Fig. 4). They included the prothoracic tergal sclerite and the verrucae bearing spinelike setae. Each seta arose from a cuplike pit known as a setal alveolus (Fig. 5). They showed low retention of conidia. Second, the greater part of the cuticle, flexible and elastic, bore thornlike processes. This structure appeared well adapted to retain numerous conidia (Fig. 6). Finally, the abdominal legs covered with a complete circle of well-developed hooks surrounded by several circles of smaller ones also trapped numerous conidia (Fig. 7).

Vineyard study. In the vineyards, *B. cinerea* was isolated from the larvae of the second generation. Larvae were captured in clusters of grapes, some of which developed early Botrytis rot. The mean percentages of insects from which *B. cinerea* was isolated and corresponding 95% confidence intervals were as follows: 95% (83–99%) in 1987 and 35% (23–48%) in 1988. When observed with SEM (Figs. 8–10), numerous conidia distributed as previously described were revealed on the segment cuticle (Fig. 8). The conidia appeared lightly collapsed and wartless. Warty and globose *Penicillium* type smaller spores 3 μm in diameter, were also associated with *L. botrana* (Fig. 9). Thin bonds about 0.1 μm diameter could be seen between spores (Fig. 10). The bonds sometimes came in the form of a network.

Observations of *B. cinerea* in the gut. Evidence of the presence of *B. cinerea* in the gut was obtained by using laboratory-grown larvae fed on *B. cinerea* for 24 hr. Light microscopic observation of the midguts showed numerous conidia and pieces of conidiophores (Fig. 11). The length of the pieces of conidiophores ranged from about 100 to 400 μm. This fungal material was well

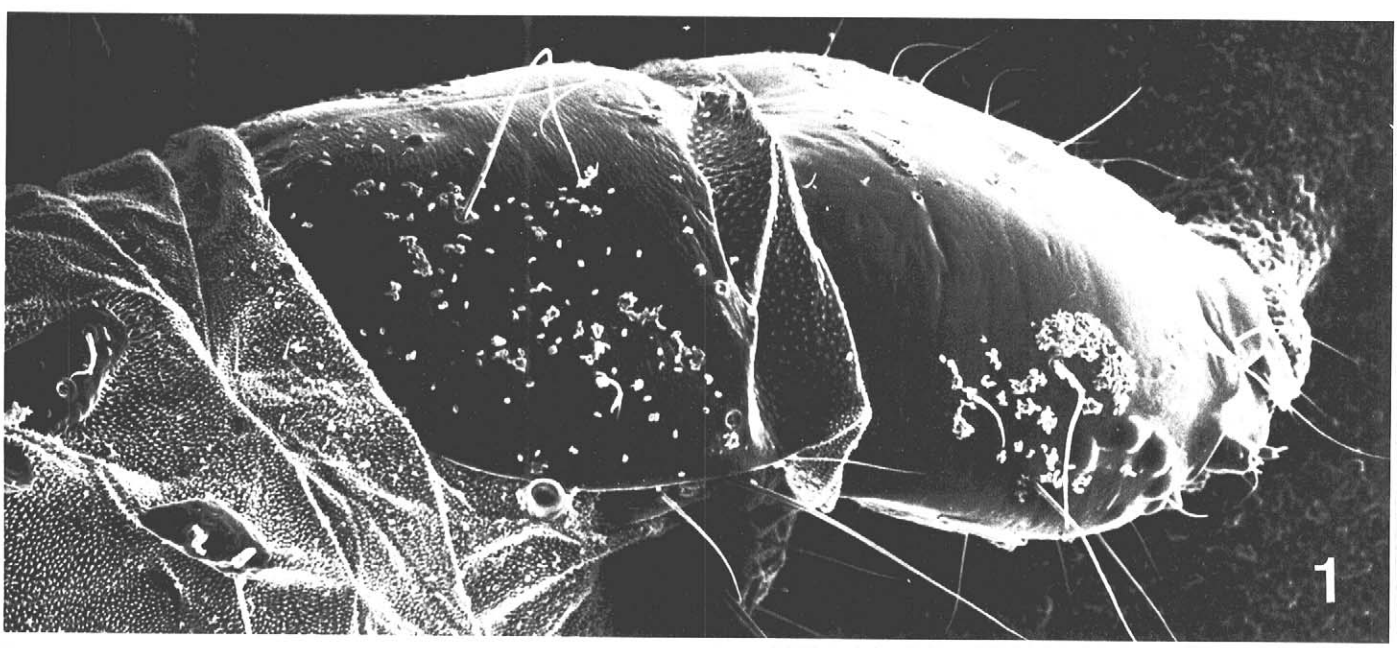
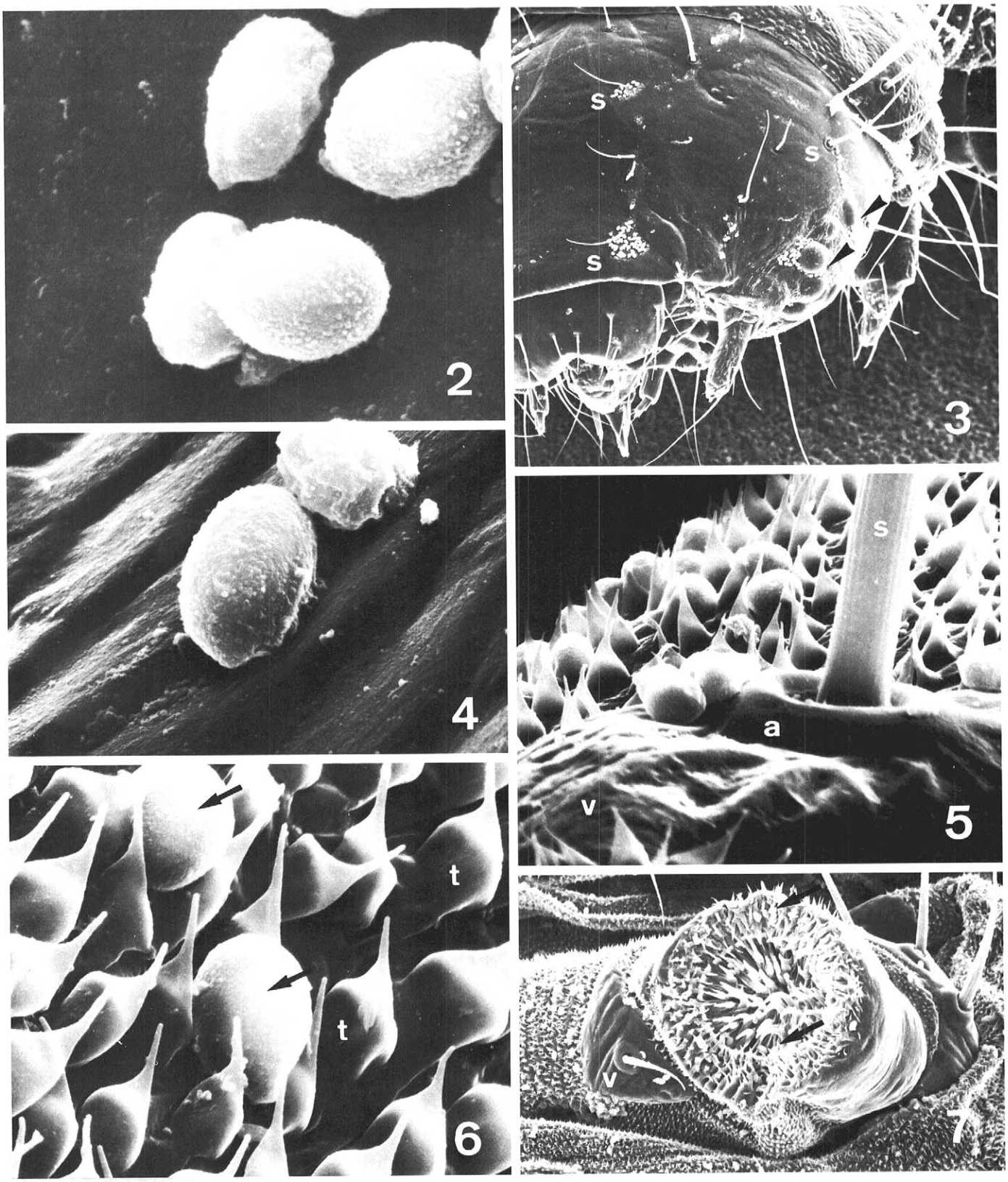
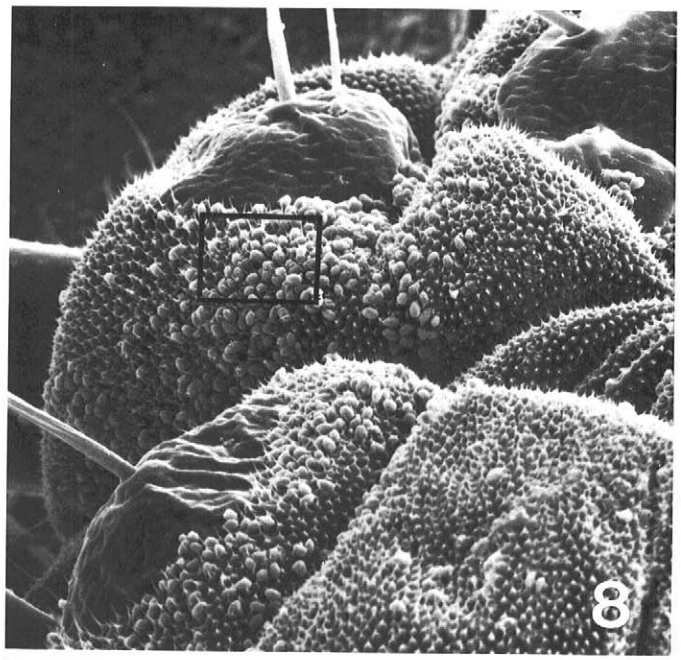


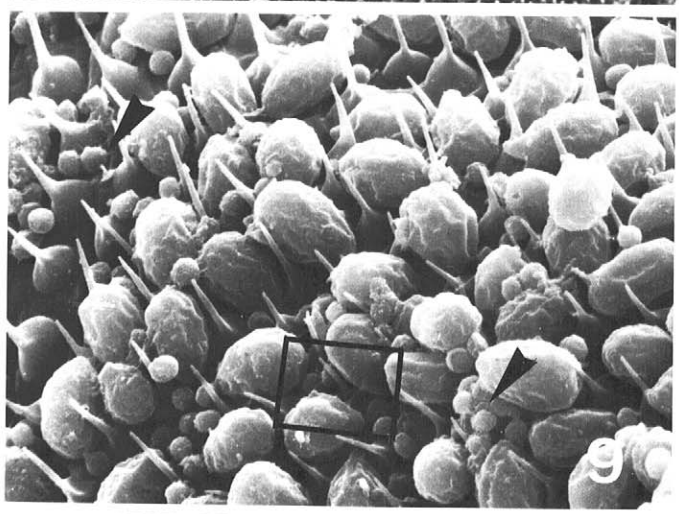
Fig. 1. Scanning electron micrograph showing numerous conidia of *Botrytis cinerea* on the head, prothoracic, and mesothoracic segments of a *Lobesia botrana* larva fed for 1 hr on a sporulating culture. ×100.

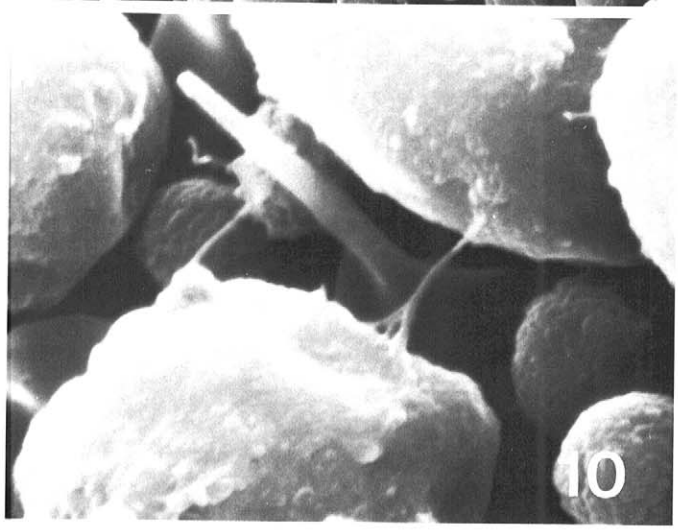
delimited inside the gut (Fig. 12). SEM revealed the presence of fragments of the peritrophic membrane enclosing feces from laboratory-grown larvae fed on *B. cinerea* for 24 hr. The ingested spores were covered by the membrane as by a fine veil. Its surface appeared smooth without conspicuous external ornamentation. The same phenomenon was observed on feces of vineyard larvae (Fig. 13). This membrane as observed by TEM consisted of several layers and ranged from 150 nm to 1 μm in thickness (Fig. 14). Just below it, typically shaped conidia were observed. Their walls ranged from 0.2 to 0.4 μm, and most were 0.25 μm in thickness.



Figs. 2–7. Scanning electron micrographs showing conidia of *Botrytis cinerea* on the cuticle of laboratory-grown larvae of *Lobesia botrana* brought into contact with the fungus for 1 hr. **2 and 3,** Conidia on the smooth head capsule around stemmatae (arrows) and at the base of the spinelike setae (s); **4,** on the ridged prothoracic tergal sclerite; **5,** near an alveolus (a) beared on a verruca (v). **6,** Conidia (arrows) trapped in the thornlike processes (t) of the major part of the flexible and elastic cuticle. **7,** An abdominal leg with numerous conidia (arrows) in the complete hook circles. Magnifications: **2,** ×4,000; **3,** ×100; **4,** ×4,000; **5,** ×1,500: **6,** ×3,500; **7,** ×200.







Figs. 8–10. Conidia of *Botrytis cinerea* trapped on the prothoracic and mesothoracic segments of a wild larva of *Lobesia botrana*. **9** is the enlargement of the inset area in **8**. Note also the presence of smaller globose spores (arrows). **10** is the enlargement of the inset area in **9** showing thin bonds between conidia of *B. cinerea*. Magnifications: **8**, ×200; **9**, ×1,200; **10**, ×8,000.

The inner layer of the conidia wall and the peritrophic membrane were PATAg positive indicating their polysaccharidic composition.

The localization of spores and conidiophores in the midgut was observed with light microscopy and is summarized in Table 1. There was no trace of the fungus in guts of the laboratory-grown larvae when not fed on *B. cinerea*. When the larvae were fed 15, 30, and 60 min on sporulating cultures and then dissected, the conidia were found in the first third, half, and three-quarters of the midgut, respectively. For feeding periods of 1 or 2 hr, no significant differences ($P = 0.05$) were observed in the mean localization of the conidia. Conidia could lack from the last quarter of the midgut because of molding and ejection of feces after the first three quarters of the midgut. So, on average, the rate of 100% was not reached. Therefore, 1 hr was considered as the time required for the conidial passage through all the digestive tract. A similar phenomenon was observed concerning the pieces of conidiophores. But in a length of midgut portion containing fungal material, conidiophores were found only in the first half.

Isolation of viable *B. cinerea* from larval feces. None of the 95 feces originating from larvae that were not fed on *B. cinerea* were infected by the fungus. For similar feces brought into contact with *B. cinerea* for 5 hr, the mean infection rate was 60.5% (±11% at $P = 0.05$). Surface-sterilization of such controls reduced the mean infection rate to 2.6% (±2.2% at $P = 0.05$). However, surface-sterilized feces originating from larvae fed on *B. cinerea* were infected on average at the rate of 58.1% (±7.6% at $P = 0.05$). In this last case, the surface-sterilization is considered to be a safeguard against the potential superficial infections of the feces due to conidia present on the larval cuticle. This demonstrates that there was viable fungal material inside these feces. The peritrophic membrane probably constituted a barrier protecting this internal *B. cinerea* from the sterilization.

Germination ability of conidia isolated from parts of the gut. The mean germination rate of conidia from pure culture on which larvae were fed, was 87.5% on agar medium. The mean germination rates of conidia from stomodaeum and proctodaeum were 90.4 and 91.2%, respectively. All three percentages were not significantly different at $P = 0.05$. Therefore, we assessed that the germination ability of conidia is not modified by the passage through the digestive tract.

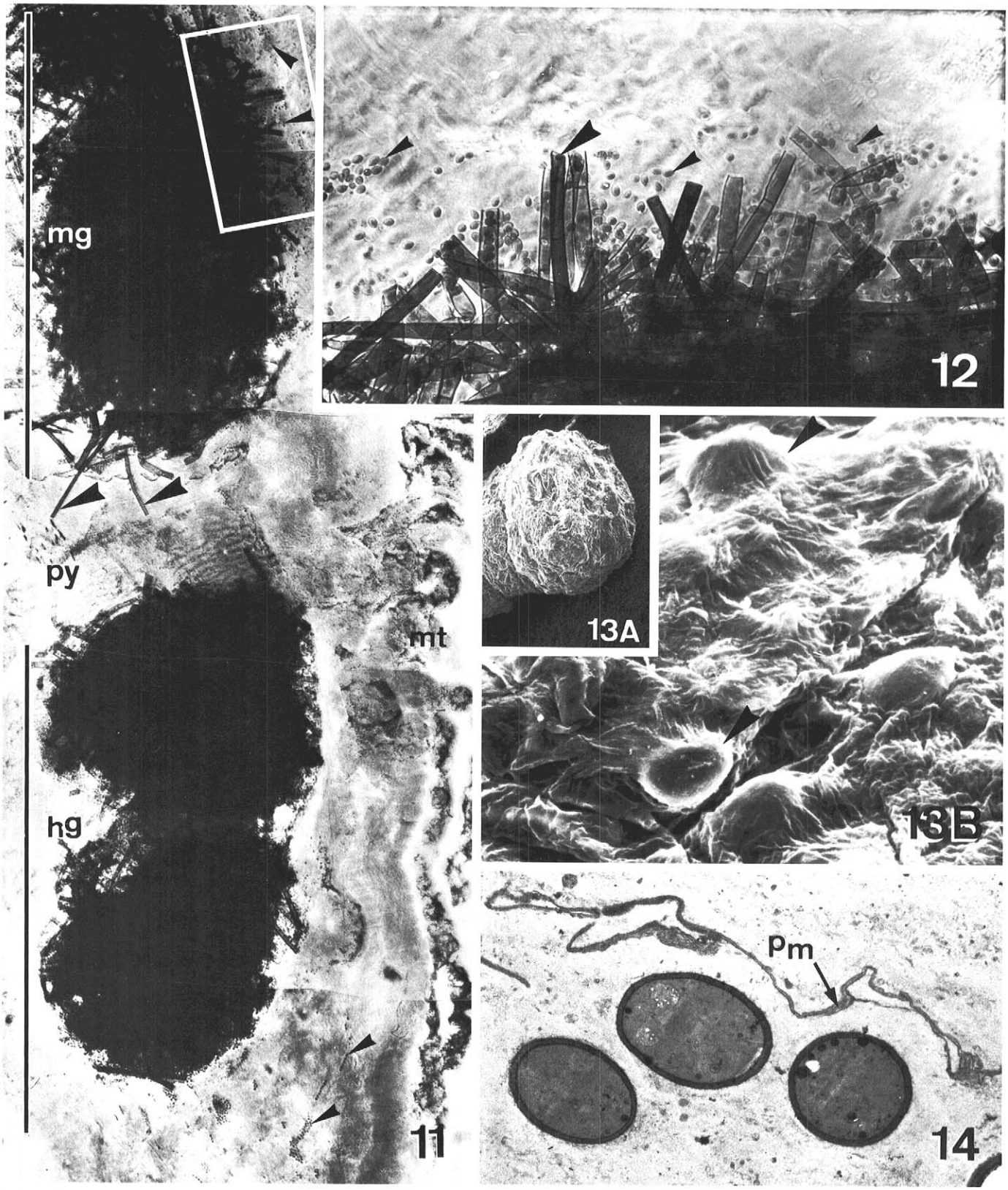
DISCUSSION

The potential role of the larvae of *L. botrana* as a vector of *B. cinerea* was demonstrated. The larvae can carry the conidia both on and in their bodies. In the vineyards both conidial association and ingestion can occur because spores were found on the larval surface and in the feces.

The presence of numerous conidia, particularly on the flexible and elastic cuticle parts bearing thornlike processes provided evidence that such ornamentations are closely involved in conidia trapping. However, the presence in the vineyards of other smaller spores at the same place show that other phenomena may also occur, permitting the adhesion of spores to the cuticle. The thin bonds observed between spores give support to this hypothesis. The occasional network structure of the thin bonds and their diameter (0.1 μm) were very similar to the 1→3, 1→6 β D glucan produced by *B. cinerea* in the infected berries between the epidermal and pulp cells (5). Nevertheless, this relationship between insect and fungus may be casual, as reported by Ingold (10) in the case of the spread by wasps of *Monilinia fructigena* (Fr.) Westend. on apples. Similarly, the conidia are dry and not slimy as in most insect-dispersed fungi, and may also be spread by wind. On the other side, this association is also profitable for *L. botrana*. A faster larval growth is, in fact, reported when larvae were fed on apples or grapes infected by *B. cinerea* (17).

The germination ability of the conidia does not seem modified by the passage through the digestive tract. It would appear, then, that no residual material of the larval excretion or digestion have an inhibitory effect on conidial germination. The possibility of

the digestion of a certain number of spores cannot be ruled out. Finally, the time of 1 hr required for conidia passage through the digestive tract appears normal as compared with food movement in larvae of other insects, e. g., *Prodenia eridania* at 2–7.5 hr or *Tribolium castaneum* at 40–45 min to 240–258 min (9). From this, it follows that the feces left on the surface of the attacked berries or inside the larval galleries may be centers of infection by the fungus.



Figs. 11–14. 11 and 12, Light micrographs showing conidia (smaller arrows) and pieces of conidiophores (larger arrows) in the gut of a *Lobesia botrana* larva fed on *Botrytis cinerea* for 24 hr. hg = hindgut, mg = midgut, mt = malpighian tubes, py = pylorus. **12** is the enlargement of the inset area in **11** showing the fungus well delimited inside the midgut. **13,** Scanning electron micrographs of a vineyard feces collected in a berry infected with *B. cinerea*. **A,** General view. **B,** Detail showing conidia of *B. cinerea* (arrows) covered by the peritrophic membrane as by a fine veil. **14,** Transmission electron micrograph of a feces section from a laboratory-grown larva fed on *B. cinerea* for 24 hr contrasted using the PATAg reaction. pm = peritrophic membrane. Magnifications: **11,** ×40; **12,** ×130; **13A,** ×40; **13B,** ×2,000; **14,** ×4,000.

TABLE 1. Percentage of the midgut length with conidia and conidiophores according to various feeding periods on *Botrytis cinerea*

	Feeding periods on *B. cinerea* (min)			
	15	30	60	120
Conidia	30 c[a]	48 b	79 a	68 a
Conidia and conidiophores	14 b	25 b	61 a	56 a

[a] Means within a line followed by the same letter are not significantly different at $P = 0.05$ according to Newman and Keuls' test after analysis of variance.

The question arises whether larvae influence the development of Botrytis rot by making the wounds necessary for infection and by introducing into these wounds the conidia in two main forms: embedded in the feces or free inside the galleries. This potential infection process, if occurring near the period of véraison, would be of great importance because grape berries in the stages between shatter and véraison contain substances that inhibit germination and growth of conidia of *B. cinerea* (13). The resistance principle of these young grapes is localized in the skin and is lacking in the fruit flesh (8). Therefore, the larvae of the second generation feeding on immature grapes could influence the pathogenic fungus by causing early centers of infection.

Vineyard larvae carrying conidia were captured in clusters, some of which developed early Botrytis rot. This fact supports the view that the source of inoculum responsible for the larval contamination could be the conidia produced by infected berries of the cluster or conidia deposited on the berries's surface after having been wind-dispersed. The contamination of larvae of the third generation is more probable than of the second one because of the increasing concentration of conidia in the air as the crop matures (4). The association of *B. cinerea* with grape berry moth larvae suggests that this insect plays an important role in dissemination of the disease. This role is being investigated in the vineyards near Bordeaux. If the grape berry moth is truly important in dissemination, it will then be possible to improve the control of Botrytis rot by a more effective control of the grape berry moth.

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