Transmission of *Botrytis cinerea* to Grapes by Grape Berry Moth Larvae

M. Fermaud and R. Le Menn

INRA Centre de Recherches de Bordeaux, Unité de Recherches Intégrées d'Aquitaine, SRIV, 33883 Villenave d'Ornon, France, and Université de Bordeaux 1, Département de Microscopie Electronique, 33405 Talence, France.
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ABSTRACT

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Larvae of *Lobesia botrana*, the grape berry moth, increase the severity of gray mold on grapes (cv. Sauvignon). The role of larval wounds on the colonization of immature berries by *Botrytis cinerea* was studied with scanning electron microscopy. On vineyard berries, emergence of conidiophores is favored at the entrance of larval galleries, and fungal development is enhanced at superficial wound sites. A greenhouse trial showed that conidia were introduced inside larval galleries by larvae externally contaminated by the fungus. The germination of conidia and resulting mycelial colonization followed on the inner surfaces of galleries. The role of transport of viable conidia by larvae was also assessed under field conditions. On immature berries, the artificial supply of viable conidia on the cuticle of second-generation larvae caused a significant increase in the percentage of larval injuries infected by *B. cinerea* (20% for clusters with normal compactness and 10% for thinned clusters after 15 days of larval presence). As for damage to ripe berries, third-generation larvae carrying viable conidia caused a 2.4% increase in disease severity at harvest as compared with larvae carrying dead conidia.

Additional keywords: bunch rot, epidemiology, tortricidae, vection, vine.

Bunch rot caused by *Botrytis cinerea* Pers.:Fr. is a serious disease of grapes (*Vitis vinifera* L.). Botrytis bunch rot of grapes in the Bordeaux vineyards reduces harvest yield and quality and markedly reduces the quality of wines, especially red (16). Loss caused by Botrytis bunch rot varies from one year to the next, depending primarily on weather conditions in late summer and autumn. In many European vineyards, infection of grapes by *B. cinerea* has been repeatedly associated with grape berry moth damage (3,9,15,18,23,24). The two European species of grape berry moths, *Lobesia botrana* Den. & Schiff. and *Eupoecilia ambiguella* Hb. (Lepidoptera: Tortricidae), are involved in the infection process. A similar phenomenon has also been reported in Hungary with *Argyrotaenia pulchellana* Haw., a polyphagous moth that can infest grapevines (25).

L. botrana is mainly distributed in the southern European vineyards, where it generally completes three generations a year. The first-generation larvae attack the flowers and can directly cause losses in harvest yield. The larvae of the second generation feed on immature grapes, promoting infection by *B. cinerea* in midseason. A positive correlation has been found in the Bordeaux region between the number of larvae and the number of primary infections on the berries (7). The natural contamination of second-generation larvae by *B. cinerea* ranged from 35 to 95% (8). The third-generation larvae damage the ripe berries before harvest, predisposing the fruit to invasion by *B. cinerea*, as the concentration of conidia in the air increases (4). In France, disease severity increased from 5% rot per cluster in noninfested grapes to almost 50% in grapes infested by larvae of the last two generations (9).

Insects influence the development of plant diseases by disseminating pathogens (26) and opening sites through which inoculum can enter (2,13). The latter hypothesis was suggested for *L. botrana* by Agrios (1). Elsewhere we have shown that viable conidia of *B. cinerea* were carried externally or internally by larvae of *L. botrana* (8). This study was undertaken to investigate in vineyards the epidemiological consequences of transport of conidia by both second- and third-generation larvae of *L. botrana*. It was necessary to determine whether the inoculum of *B. cinerea* carried by *L. botrana* larvae enter the grapes at the time of larval penetration and cause them to rot. This study should help to establish the role of grape berry moth larvae as a vector of the gray mold pathogen.

MATERIALS AND METHODS

B. cinerea. An isolate (R1) of *B. cinerea* isolated in 1982 from the cultivar Sauvignon in a vineyard near Bordeaux was used. Stock cultures were maintained on 1.5% malt agar. For use in this study, abundantly sporulating cultures were obtained after a 1-wk incubation at 12:12 (light-dark) photoperiod at 20 C.

L. botrana. The larvae completed their growth on a semi-synthetic diet in the laboratory using breeding methods previously described (21). Larvae in their fourth and third instar were used for the field trials concerning the second and third generation,

respectively. The larvae were artificially contaminated with *B. cinerea* by 1-h contact with the surface of petri dishes containing sporulating *B. cinerea* (8).

Scanning electron microscopy. Grape berries of the cultivar Sauvignon attacked by second-generation *L. botrana* larvae were collected in the INRA experimental vineyard near Bordeaux at the end of July 1987, 10 days before the onset of *véraison* (beginning of ripening), at Eichhorn and Lorenz's growth stage 35 (6). Two kinds of berries were separated visually: those developing early Botrytis rot and those not developing rot. In order to expose the inside of the larval galleries, the berries were dissected with a sterile scalpel. The samples were individually fixed for 24 h in a solution of 6.25% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4. They were then rinsed three times with fresh buffer, dehydrated through graded ethanols, and CO_2 critical-point dried. They were mounted on aluminum stubs, coated with gold palladium alloy by sputtering, then coated with carbon by vaporization on a rotating stage. The observations were made with a JEOL 840 A scanning electron microscope (SEM) operated at 10, 20, or 30 kV.

Transport and depositing of conidia of *B. cinerea* inside the larval galleries. Twenty grape clusters of the cultivar Merlot were grown in the greenhouse out of season under conditions of low air concentration of *B. cinerea* conidia. Each cluster was infested with 10 third-instar larvae of *L. botrana* on 4 February 1991, at the "beginning of berry touch" stage, Eichhorn and Lorenz's growth stage 33 (6). Half of the clusters were infested with larvae contaminated with the fungus as mentioned above. The other 10 clusters were infested with uncontaminated larvae and used as controls. After infestation, the clusters were enclosed in plastic bags. Five berries damaged by contaminated larvae were randomly collected after 4, 7, and 11 days, prepared, and observed with SEM as mentioned above. Moreover, to determine if the larval galleries were contaminated by *B. cinerea*, 11 days after inoculation 22 berries damaged by contaminated larvae and 115 damaged by uncontaminated larvae were studied as follows. Under sterile conditions, the berry skin was removed with a scalpel. The berry flesh, including larval galleries, was then plated on 1.5% malt agar with 250 mg/L of chloramphenicol. *B. cinerea* was identified with the aid of a stereomicroscope after 7 days of incubation at 20 C.

Effect on disease incidence of *B. cinerea* transported by second-generation larvae. The effect of transport of conidia by second-generation larvae on disease incidence was studied in 1987 and 1988. Experiments were carried out in 0.5 ha of a Sauvignon vineyard of low-wire cane and spur (double guyot)–trained vines planted at a standard 1.10 × 1.80 m spacing located near Bordeaux.

In 1987 and 1988, 95 and 125 vines, respectively, were randomly selected. For each vine, four first-rank (lowest) clusters of shoots situated near the trunk were selected. According to Huglin (11), these clusters are similar in compactness. For 190 clusters in 1987 and 300 in 1988, the compactness was reduced by half by randomly removing grapes from the bunch with scissors at the "beginning of berry touch" stage. For these thinned clusters, the fruit set ratio, calculated as the number of berries divided by the number of flowers per cluster, was reduced from 28 to 15% in 1987 and from 58 to 30% in 1988.

The clusters were inoculated on 20 July 1987 and 14 July 1988 at the "beginning of berry touch" stage, when natural second-generation larvae were in their fourth instar. Bunches of grapes were inoculated, at the rate of two larvae per cluster, with live larvae contaminated with viable conidia, live larvae not contaminated with *B. cinerea*, or dead larvae contaminated with viable conidia. This last method of inoculation was done by puncturing the berry surface with a needle and plugging the wound with a contaminated, then mechanically killed, larva. This amounted to inoculation of *B. cinerea* with the same quantity of spore inoculum as that carried by live larvae. Live larvae were placed in the clusters using a paint brush. In 1987, for each level of compactness, 80, 30, and 80 clusters, respectively, were inoculated using the methods above. In 1988, 125, 125, and 50 thinned clusters and 75, 75, and 50 control clusters, respectively, were inoculated.

In 1987 and 1988, three assessments of larval infestation and disease severity were made: first, 15 days after inoculation, at the onset of *véraison*; second, 30 days after inoculation; and third, 60 days after inoculation, at harvest. With regard to inoculations with live larvae, larval infestation was determined by examining the clusters for visual evidence of larval damage (one injury being defined as damage caused by one larva on about three berries) and larvae. At the place where dead larvae were inoculated and where a live larva caused an injury, the occurrence of rotted berries was noted. Percentage of larval injury with *Botrytis* infection was determined for each assessment date.

Effect on disease severity of *B. cinerea* transported by third-generation larvae. The effect of conidia transported by third-generation larvae on disease severity was studied in 1990 in the same plot as for the previous studies. The modes of inoculation were arranged in a randomized block design, and each block of seven adjacent vines was replicated five times. Treatments were applied at random to experimental units consisting of 14 randomly selected healthy clusters per block. The clusters were inoculated on 10 August 1990, at the "beginning of *véraison*" stage. Bunches of grapes were inoculated by the three above-mentioned methods except that the second one was replaced by inoculation with live larvae contaminated by dead conidia of *B. cinerea*. The conidia had been killed by heating petri dishes containing sporulating *B. cinerea* at 53 C for 15 h. In Jarvis (12) and in our unpublished observations, this exposure of dry conidia to 53 C results in a sharp drop in germination near the thermal death point. Additional clusters were tagged as uninoculated controls.

Grape clusters were examined at maturity (on 30 August). Severity ratings were determined by counting rotted berries per cluster and converting these figures to a percent rot per cluster based on the average number of berries per cluster.

Analysis of data. In 1987 and 1988, for each date the mean percentages were compared in pairs at $P = 0.05$, using the chi-square test. In 1990, the numbers of rotted berries were transformed using the $\sqrt{x} + \sqrt{x+1}$ transformation to stabilize variances. The means were compared at $P = 0.05$ using Newman and Keuls's test (19). SAS-STAT (17) and STAT-ITCF (20) were used for statistical analysis.

RESULTS

Infection of *L. botrana*-damaged immature berries collected in the vineyard. When observed with SEM, the surface of immature berries damaged by *L. botrana* larvae showed two types of wounds resulting from bites: areas of superficial bites and larval entrance holes (Fig. 1A). These superficial bites affected up to three cellular strata.

As for berries free of apparent infection by *B. cinerea*, a few conidia could be seen in the areas where the skin was undamaged by the larvae. Microscopic examination provided no evidence that *B. cinerea* conidia were present at the site of the larval injuries.

On visibly diseased berries, conidia could be seen in the areas where the skin was undamaged by the larvae. The areas where the skin had been cut by the larvae showed increased fungal growth (Fig. 1B). In these areas numerous conidia and emerging hyphae were observed (Fig. 1C). On the edge of the entrance holes of larval galleries numerous conidia, mostly aggregated with fungal hyphae and larval silks, were present, as they sometimes were on conidiophores (Fig. 1D). Inside the larval galleries, numerous conidia were also deposited on the inner walls or on the surface of the feces left inside the galleries (Fig. 1E and F). Moreover, a sizable mycelial colonization occurred on the walls of larval galleries. Germination of conidia was observed at this site (Fig. 1G).

Infection of greenhouse berries damaged by laboratory-grown larvae carrying *B. cinerea*. Eleven days after inoculation, none of the 115 berries with galleries created by uncontaminated larvae were infected by *B. cinerea*. As for berries damaged by contaminated larvae, the mean percentage of injured berries from which *B. cinerea* was isolated was 59.1%, with the corresponding 95%

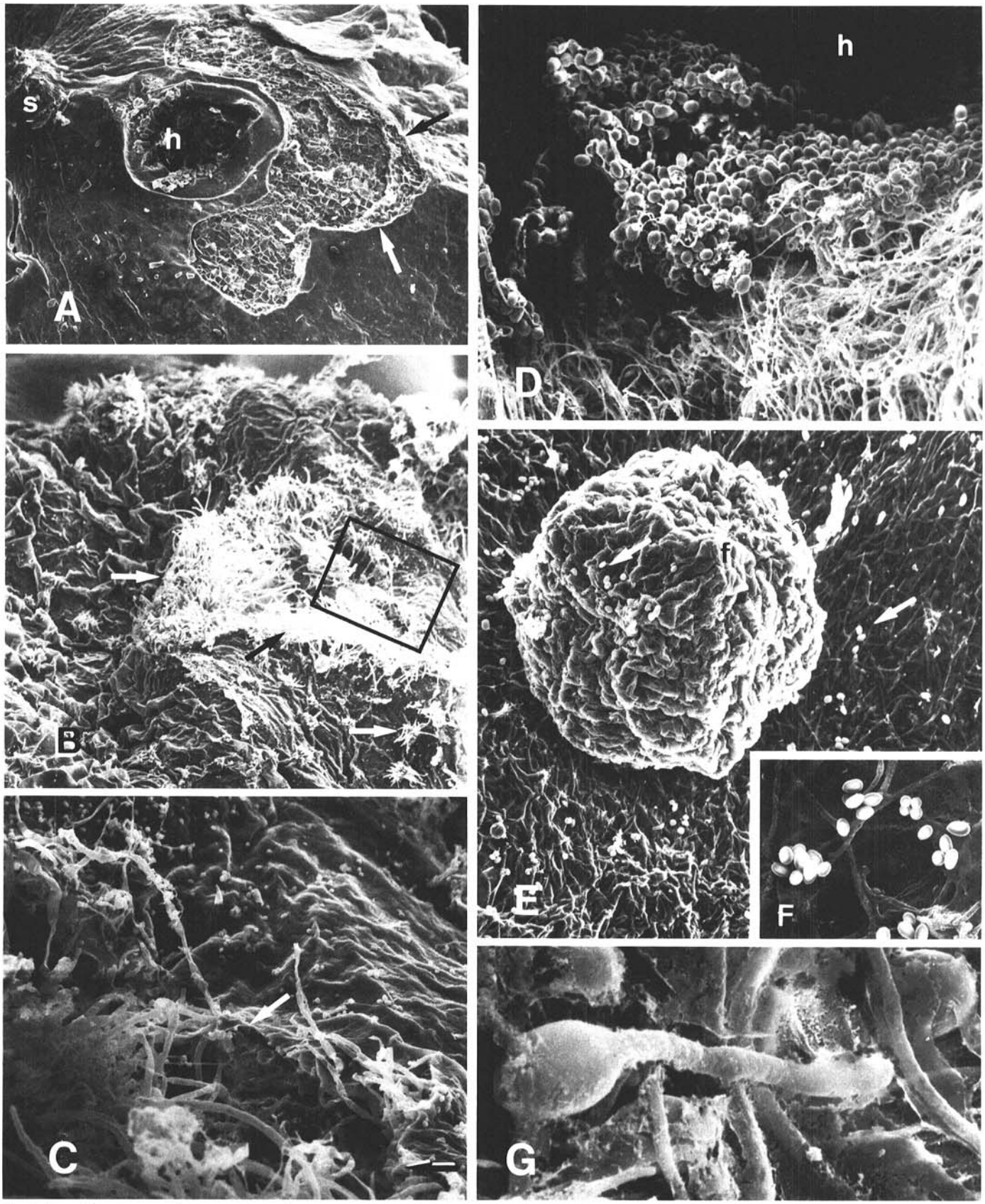

Fig. 1. Scanning electron micrographs showing *Botrytis cinerea* in immature vineyard berries damaged by second-generation larvae of *Lobesia botrana*. **A,** Larval entrance hole (h) and superficial bites (arrows) affecting the first cellular strata; s = stigma (×20). **B,** Skin area damaged by the larvae, showing an increased *B. cinerea* development (arrows) (×30). **C,** Enlargement of the inset area in **B** showing an accumulation of conidia (lower left) and the emergence of hyphae from where the skin was cut by the larvae (arrows) (×110). **D,** Conidiophore on the edge of a larval entrance hole (h) (×280). **E,** General view (×130), and **F,** detail (larval gallery wall) (×350), of *B. cinerea* conidia (arrows) and hyphae inside a larval gallery on a feces (f) and on the inner wall. **G,** Germinated conidium of *B. cinerea* on a larval gallery wall (×2,000).

confidence interval (38.5–79.6%).

B. cinerea development was observed with SEM (Fig. 2) inside the larval galleries created by contaminated larvae. Four days after inoculation, *B. cinerea* had already intensively colonized the walls of the galleries, especially at the bottom of the galleries (Fig. 2A). Figure 2B shows that conidial presence and germination was also observed there. After 7 days of larval presence, a sizable mycelial growth occurred both inside and at the entrance of the larval galleries (Fig. 2C). However, no germinated conidia could be seen at these sites. As shown in Figure 2C, numerous straight silk fibers, about 1 μm in diameter, were produced by the grape berry moth larvae. Eleven days after inoculation, mycelial mats invading the galleries walls were much thicker than previously seen (Fig. 2D). Hyphae then appeared to be embedded in a matrix linking them in a layer arrangement.

Effect of *B. cinerea* transported by second-generation larvae on disease incidence. Wound inoculations with dead larvae contaminated with viable conidia always caused infection by *B. cinerea* as early as 15 days after inoculation (beginning of *véraison*).

In 1987, the inoculation with 220 live larvae resulted in 52 larval injuries in the thinned clusters and 77 larval injuries in the control (nonthinned) clusters. So, 23.6 and 35% of the larvae survived in the thinned and control clusters, respectively. In 1988, 131 larval injuries were observed in control clusters and 184 in thinned clusters (i.e., 43.7 and 36.8% of the 300 and 500 live larvae inoculated).

Figure 3 shows the mean percentages of larval injuries infected by *B. cinerea*. In 1987 and 1988, as early as 15 days after inoculation, more than 50% of the larval injuries were infected by *B. cinerea*. The percentage of infected larval injuries increased when *B. cinerea* conidia were borne on the larval cuticle. The increase was at least 20% for clusters with normal compactness and 10% for thinned clusters. The differences were significant at $P = 0.05$ in all cases except for the thinned clusters in 1988 (X^2 for control and thinned clusters = 5.7 and 4.2, respectively, in 1987, and $X^2 = 8.2$ and 2.1, respectively, in 1988). Thirty days after inoculation, the corresponding increases were lower and not significant at $P = 0.05$ (X^2 for control and thinned clusters = 0.3 and 3.7, respectively, in 1987, and $X^2 = 0.7$ and 0.05, respectively, in 1988). At the time of the first two assessments (15 and 30 days after inoculation), in both types of clusters, the presence of conidia on the larval cuticle resulted in an increased infection rate of the larval injuries. Sixty days after inoculation, at harvest, the favorable effect of larval contamination with *B. cinerea* was no longer perceptible. The differences were not significant at $P = 0.05$ (X^2 for control and thinned clusters = 0.01 and 0.04, respectively, in 1987 and $X^2 = 0.30$ and 0.31, respectively, in

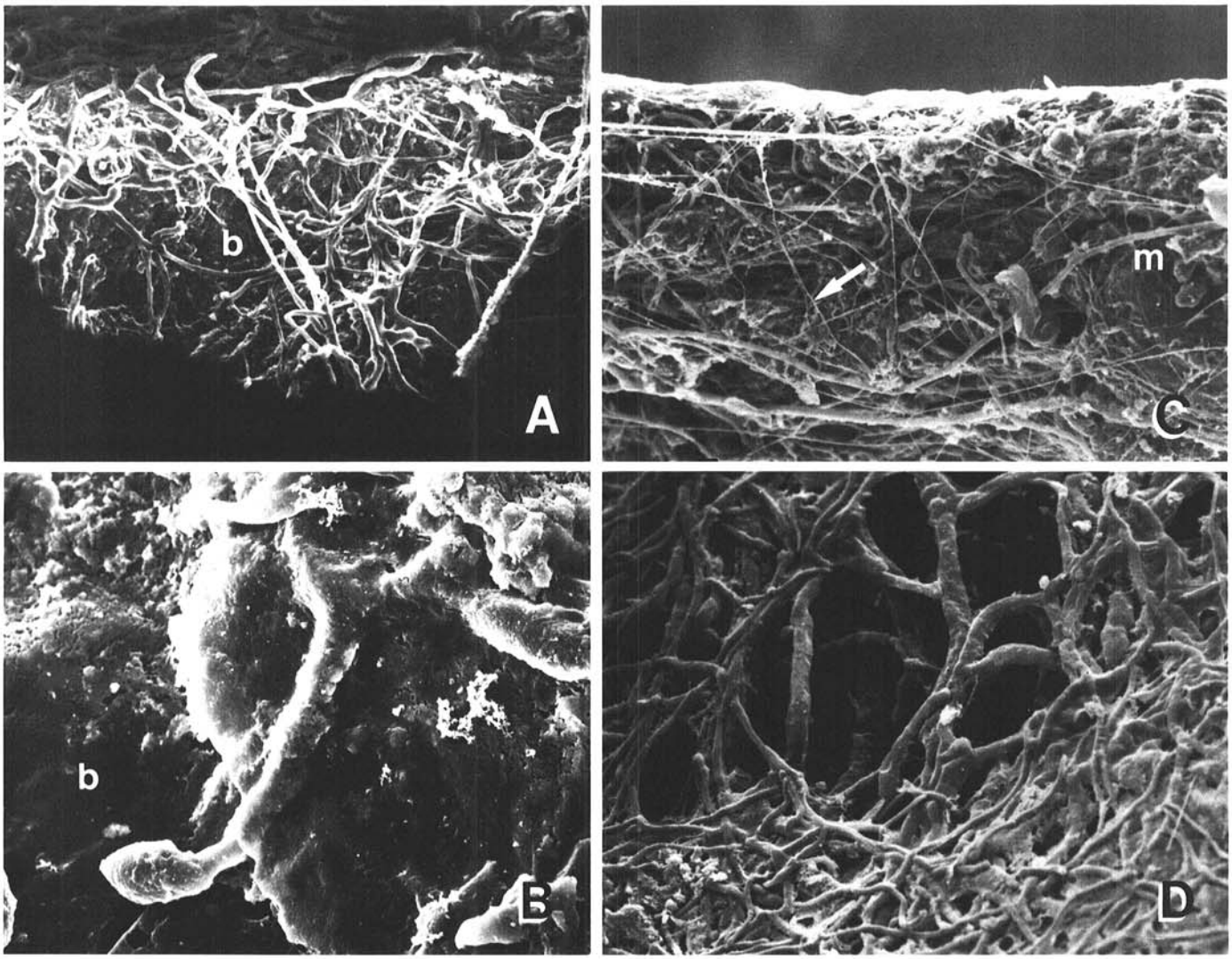

Fig. 2. Scanning electron micrographs of *Botrytis cinerea* in immature greenhouse berries inoculated with laboratory-grown larvae of *Lobesia botrana* contaminated with the fungus. **A** and **B,** Four days after inoculation at the bottom of the gallery wall (b): *B. cinerea* colonization and a germinated conidium (×280 and ×1,640, respectively). **C,** Seven days after inoculation: *B. cinerea* colonization on the edge of the entrance hole of a larval gallery, larval silk fibers (arrow), and mycelium (m) (×320). **D,** Eleven days after inoculation: mycelial mats at the bottom of the gallery wall (×450).

1988). The larval injuries were then infected by the fungus at rates ranging from 85 to 100%.

In these field trials, the increasing compactness of the clusters favored *B. cinerea* infection especially in the cases of injuries caused by the uncontaminated larvae in 1987 and by the contaminated ones in 1988. Nevertheless, the effect of compactness remained slight as compared to the effect of the presence of *B. cinerea* conidia on the larval cuticle.

Effect of *B. cinerea* transported by third-generation larvae on disease severity. In 1990, Botrytis bunch rot was relatively low in severity, as indicated by the mean percentage of rotted berries per cluster (1.68%) in the noninfested clusters of the cultivar Sauvignon (Table 1). All of the inoculation experiments led to a significant increase in disease incidence at harvest. The severity of Botrytis rot was significantly ($P = 0.05$) increased to almost 4% because of inoculations with live larvae carrying conidia killed by heat or with viable conidia borne on dead larvae. Maximum disease severity (6.43%) resulted from inoculation with live larvae carrying viable conidia. This rate was significantly greater than the preceding ones at $P = 0.05$.

DISCUSSION

Data from field trials show that damage by the second- and third-generation larvae of the grape berry moth (*L. botrana*) predisposes grapes to invasion by *B. cinerea* (3,9,18,24). Attempts have been made to relate severity of gray mold in field plots with mean levels of larval infestation. M. Fermaud, in a 3-yr study at Bordeaux, found a significant positive correlation between various levels of second-generation larvae of *L. botrana* and the number of early centers of *B. cinerea* infection on immature grapes (7).

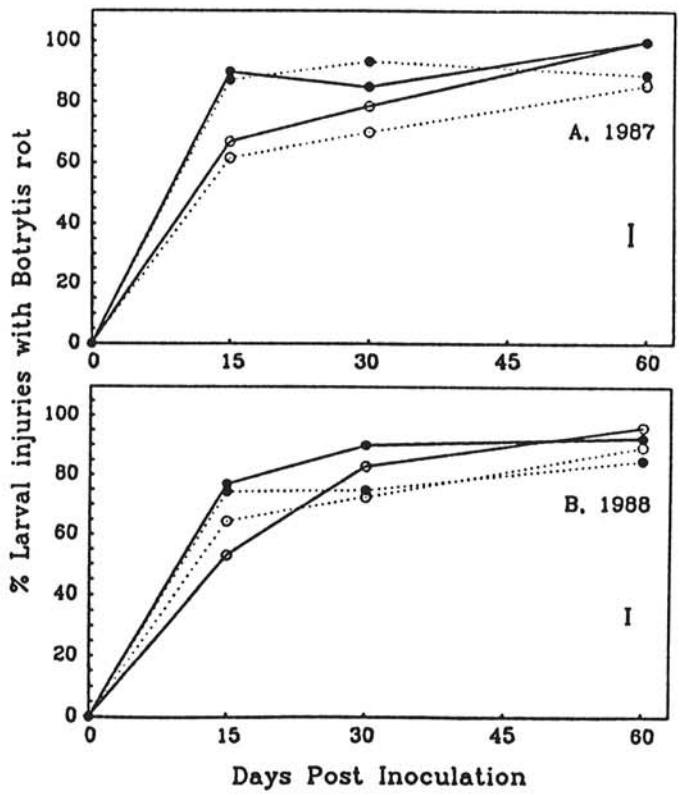

Fig. 3. Effect of contamination with *Botrytis cinerea* of second-generation larvae of *Lobesia botrana* on gray mold development. Inoculations were undertaken under field conditions on 20 July 1987 and 14 July 1988. Laboratory-grown larvae in their fourth instar were artificially contaminated with viable conidia (●) or uncontaminated (○). Two types of Sauvignon clusters were used: normal in compactness (solid line) or thinned clusters whose compactness was reduced by half (dotted line). In 1987 and 1988 a total, respectively, of 110 and 150 control clusters and 110 and 250 thinned clusters were inoculated with two larvae per cluster. The vertical bar represents the overall standard error of the mean: 7.5% (1987) and 4.5% (1988).

The microscopic examination presented here of *L. botrana*-damaged berries collected in a Bordeaux vineyard before *véraison* shows that numerous conidia were present inside the larval galleries. This provides evidence that, as purposed by Agrios (1), the holes created by the grape berry moth larvae serve as ports of entry for the fungus. Moreover, the larval injuries on the surface of the berries favored the emergence of conidiophores, especially at the entrance of the larval galleries, and fungal development is enhanced at the site of superficial bites. The question arises whether conidia were deposited on the walls of the gallery after wind dispersal or were introduced by the larvae. This last hypothesis is substantiated by the results of our greenhouse trial in the absence of aerial contaminations with conidia. *B. cinerea* conidia carried by *L. botrana* larvae enter the grapes at the time of larval penetration and are deposited on the walls of the larval galleries. Our results also show that conidia can germinate at this site, in contact with the flesh of immature berries before *véraison*. This result is consistent with those indicating that grape berries in the stages between shatter and *véraison* contain substances that inhibit germination and growth of *B. cinerea* conidia (14), and that resistance is lacking in the berry flesh (10). Under these greenhouse trial conditions, this infection process resulted in a dense mycelial colonization on the surface of the galleries walls as early as 7 days after larval presence. This may explain why, from that time on, conidia of *B. cinerea* could no longer be seen with SEM. The embedding of hyphae in a matrix 11 days after inoculation compares with the aspect of (1→3),(1→6)-β-D-glucan produced by *B. cinerea* hyphae when observed with SEM in the infected berries between the epidermal and pulp cells (5).

As for the damage of berries before *véraison* by second-generation larvae, our field trials at Bordeaux show that, for the two seasons under review, transport of viable conidia by the larvae favors the outbreak of the disease. Fifteen days after inoculation, gray mold broke out in at least 50% of the injury sites caused by larvae not carrying *B. cinerea*. This confirms that damage by the grape berry moth larvae predisposes berries to invasion by *B. cinerea*. However, the possibility of contamination of these larvae with vineyard conidia after inoculation cannot be ruled out. In any case, the artificial supply of viable conidia on the larval cuticle caused a significant increase in the percentage of larval injuries showing Botrytis rot symptoms 15 days after inoculation. The effect of the contamination of larvae was noticeable up to 1 mo of larval presence (i.e., until *véraison*). Thus, the contamination of larvae appears to be of great importance before *véraison* when the concentration of *B. cinerea* conidia in the air is still relatively low (4). On the other hand, at this stage the contaminated larvae did not cause early centers of infection in 100% of cases (90% at the maximum in Sauvignon clusters with normal tightness) as occurs for wound inoculations with the same quantity of *B. cinerea* inoculum brought on dead larvae. It follows that a certain number of spores borne by larvae

TABLE 1. Field transmission of *Botrytis cinerea* on Sauvignon grape clusters by third-generation larvae of *Lobesia botrana*

	Botrytis bunch rot at harvest	
Type of inoculation[w]	Incidence (%)[x]	Severity (%)[y]
Live larvae carrying viable conidia	98.58 a[z]	6.43 a
Dead larvae carrying viable conidia	92.86 a	3.93 b
Live larvae carrying dead conidia	81.92 a	3.97 b
Uninoculated clusters (control)	52.84 b	1.68 c

[w] Clusters (70 per treatment) arranged in a randomized five-block design were inoculated with laboratory-grown larvae in their third instar. In order to contaminate the larvae with *B. cinerea* conidia, they were brought into contact with a sporulating pure culture for 1 h.

[x] Percentage of clusters decaying from *B. cinerea*.

[y] Percentage of diseased berries per cluster.

[z] Means in each column followed by different letters are significantly ($P < 0.05$) different according to Newman and Keuls's test after analysis of variance.

probably are not introduced or deposited inside the larval galleries. This study also shows the favorable effect of cluster compactness on *B. cinerea* infection according to Vail and Marois (22). In both normal and thinned (in which compactness is reduced by half) Sauvignon clusters, the transport of *B. cinerea* conidia by the larvae increased disease incidence. Thus the larvae, when contaminated by conidia, were an efficient vector of *B. cinerea* even if the conditions were less favorable for infection of berries, as in thinned clusters. Therefore, it seems clear that natural contamination of second-generation larvae by *B. cinerea*, as previously reported in the Bordeaux vineyards (8), plays an important role in the epidemiology of the disease. Transport of conidia can partially account for the correlation between the number of second-generation larvae and the number of early centers of Botrytis rot (7).

With respect to damage to ripe berries by third-generation larvae, the effect of contamination with viable conidia was also of importance in our study. The artificial contamination of the larvae with dead conidia led to a 2.3% increase in disease severity at harvest. Because dead conidia occupied the cuticle ornamentations, external contamination with viable vineyard conidia was reduced. Therefore, the increase probably resulted from the infection of damaged berries with wind-dispersed vineyard conidia not being borne on the larvae. An almost twofold increase (+4.7%) in disease severity resulted from inoculation with larvae contaminated with viable conidia. This demonstrates the role of transport of viable conidia in the increased severity of gray mold caused by the larvae of *L. botrana*.

Our findings suggest that grape berry moth larvae can vector *B. cinerea* from infected to healthy grape berries. Therefore, sanitation practices to reduce gray mold in vineyards should include an effective control of the grape berry moth.

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