Persistence of *Botrytis cinerea* in Its Vector *Drosophila melanogaster*

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ABSTRACT

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Conidia of *Botrytis cinerea*, a pathogen of grape that causes bunch rot, were carried externally on the cuticle of the "fruit fly" *Drosophila melanogaster* and also might be carried internally through the digestive tract. Conidia germinated in the insect crop and developed into mycelium. Under some conditions, development of microsclerotia, which are fungal survival structures the flies can carry their entire life, may occur. *Drosophila* must be considered a plurimodal vector of *B. cinerea*, supporting nonpersistent, semipersistent, and possibly persistent transmission of *B. cinerea*.

RÉSUMÉ

Les conidies de *B. cinerea* sont véhiculées sur la cuticule de *D. melanogaster* et peuvent aussi transiter par le tube digestif. Les conidies peuvent germer dans le jabot de l'insecte et donner un mycelium qui, dans certaines conditions, se différencie en microsclérotes, formes de résistance que l'insecte peut transporter jusqu'à la fin de sa vie. La drosophile peut donc être considérée comme un vecteur plurimodal de *B. cinerea*, le transmettant par vection nonpersistante, semipersistante, et probablement par vection persistante.

Additional keywords: aseptic rearing, fruit rot, gut, histology, microbiology, Moniliales, plant pathogens.

Pre- and postharvest fungal diseases result in important reductions of fruit quality and quantity for both producers and consumers. *Botrytis cinerea* Pers.:Fr. is an economically important pathogen for a wide range of cultivated plants in many regions of the world. In the European Union, diseases caused by *B. cinerea* are particularly important in the production of peaches, citrus, and table and vine grapes. Bunch rot of grapes (*Vitis vinifera* L.), caused by *B. cinerea*, reduces the quality of wines (2). Bunch rot of grapes is influenced by climatic factors. Late-summer rainfall favors *B. cinerea* development in viticultural regions. The role of the vector *Diptera* in transmission and dissemination of fungal pathogens of plants has been studied in tomatoes (3,4), but its role is not yet sufficiently understood (1,23). *Drosophila* has long been known as a microorganism disseminator, particularly in vinification. At the end of the previous century, Pasteur's work showed the involvement of *Drosophila* spp. in dissemination of yeasts and bacteria involved in wine fermentation and alcohol acidification (22). Transmission of *Rhizopus stolonifer* by *D. melanogaster* Meig (18) in orchards in the Rhone Valley of France and transmission of *B. cinerea* by grape berry moth larvae in the French vineyards near Bordeaux (9,10,11) have been demonstrated. In both cases, spores adhere to the insect cuticle and are dispersed by feces. Our own observations and reports in the literature and by consulted wine growers suggest that *B. cinerea* is more important as a rotting agent for grapes than for peaches.

The presence of the "fruit" or "vinegar fly" *Drosophila* in vineyards, wine factories, and table grapes is commonly observed. *Drosophila* pullulation is favored by climatic factors such as summer rainfall. Capy et al. (5) have described the proliferation of *Drosophila* spp. in vineyards.

Analysis of the transmission potential of *Drosophila* for *B. cinerea* and the mechanisms involved in this transmission is of great interest. To analyze this potential in strictly controlled conditions, complementary experimental methods were used. The *D. melanogaster* 'Champetières' strain was chosen as a model because this strain is reared in aseptic conditions.

The aim of the present paper is to report the transmission mechanisms of the main grape fungus, *B. cinerea*, by the fly *D. melanogaster*, demonstrating external (cuticular) transport of *B. cinerea* conidia as well as ingestion and conidial transit through the digestive tract. We also report crop invasion by *B. cinerea*, conidial germination, hyphal development, and microsclerotium differentiation in the fly crop.

MATERIALS AND METHODS

Preliminary series of experiments. For 2 years, *Drosophila* spp. were collected from vineyards, table grapes, or wine factories from the Languedoc area of France naturally infested with *B. cinerea*. After collection, insects were transferred to sterile media or microscopically examined after dissection of the digestive tract.

Laboratory strain of *D. melanogaster*. 'Champetières,' a "germ-free" strain, was used as a wild-type stock highly stabilized by single brother-sister matings over 40 years. Larval stages were reared on David's sterile medium (8) containing 166 g of corn meal, 166 g of live baker's yeast, 30 g of Difco (Detroit) purified agar, 10 g of methyl benzoate (Nipagin) as a preservative, and up to 2 liters of distilled water. The medium was autoclaved at 121°C for 20 min. Emerging flies were isolated and reared on specially adapted CL (yeast extract) medium without preservative that contained 80 g of Difco Yeastolate yeast extract instead of live yeast. Flies were asep-

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tically reared as described by Louis et al. (18). As a rule, the *Drosophila* rearing temperature was 20°C. Sexing (male and female separation) was performed with virgin (emerging) flies. Flies used in experiments were 2 to 3 days old. *D. melanogaster* manipulations were performed in sterile conditions.

According to Graham-Smith (14), "The crop is a pedunculate sac evaginated from the oesophagus.... The sac is capable of great distention by ingested liquid food, its size being dependent upon the age and nourishment of the fly.... The crop serves to receive and store liquid food, passing it on to the ventriculus for digestion." Dissections of the digestive tract (fly crop included) were performed in sterile Drosophila Ringer's solution (21) after treatment with 3% sodium hypochlorite and rinsing of CO_2-anesthetized flies.

Fungal strains. *B. cinerea* strains C1 from Champagne and B1 and S22 from Bordeaux vineyards were generously provided by the INRA Phytopathology Research Station (Bordeaux, France). *B. cinerea* strains were cultured on potato-dextrose agar (PDA). For use in this study, cultures that sporulated well were obtained after 1 week of incubation in a 12:12 h (light/dark) photoperiod at 20°C. The flies were contaminated artificially with *B. cinerea* by rearing them in petri dishes or tubes containing sporulating *B. cinerea* for 15 to 45 min and then transferring the flies to CL sterile medium, which supports *B. cinerea* growth. *B. cinerea* was identified under a stereo microscope after 11 to 12 days of incubation at 20°C.

Gross dissemination of *Botrytis* by *Drosophila*. Experimental imagos were divided into two groups; 10 males and 10 females were reared for 12 h on a *B. cinerea* (B1 strain) culture. Each group was reared for 22 days at 20°C in successive tubes containing CL medium. The tubes were changed every 2 h for 1 day and then every 2 days. Tubes were maintained at 20°C and were examined 2 weeks later for *B. cinerea* growth.

External and internal *B. cinerea* transmission. The relative significance of external and internal fungi transmission by 'Champetières' flies was studied by several means, including scanning electron microscopic (SEM) examination of the insect cuticle, light microscopic observation of the digestive tract after dissection (more than 500 flies were examined, including preliminary experiments), and sterile culture of digestive tract contents on PDA medium.

For SEM examination, flies were allowed to feed for 1 h on tubes containing a sporulating fungal culture and then were transferred to sterile tubes that were changed every hour. These changes allowed the flies to be progressively cleared of spores by mechanical contact and by cleaning themselves. Flies were anesthetized with CO_2, killed by freezing at –20°C for 2 h, vacuum-dehydrated (lyophilized), coated with gold, and examined using a Zeiss (Oberkochen, Germany) DSM 950 SEM electron microscope operated at 10 kV.

Two types of experiments were conducted to determine whether conidia remained alive at the end of intestinal transit: feces cultivation and direct observation in fly rectums. For feces cultivation, aseptically reared *D. melanogaster* flies were fed on sporulating *B. cinerea* (B1 strain) cultures for 1 day and then were transferred to sterile medium in petri dishes. Feces were isolated with sterile glass microneedles and cultured on sterile medium. As controls, feces of aseptically reared *D. melanogaster* flies were cultured. For microscopic observation of rectums, *Drosophila* flies were reared for 12 to 24 h on sporulating *B. cinerea* (B1 strain) culture medium, aseptically dissected, and their rectums were transferred in sterile Drosophila Ringer's solution on sterile microscope slides.

Light microscope observations and micrographs were made with a Wild (Heerbrugg, Switzerland) M 20 microscope with phase contrast, photographic camera, and flash attachments. As controls, rectums of uninfected flies (aseptically reared) were examined under the same conditions. Crops were dissected after rearing flies on sporulating *B. cinerea* colonies and were examined by light microscopy under phase contrast to assess the presence and development of spores.

Quantitative study of the developmental stages of *B. cinerea* strains in the *D. melanogaster* crop. Emerging male and female flies were separated into groups of about 100 individuals each. They were kept for 1 day on a sporulating culture of B1, C1, or S22 *B. cinerea* strains and then were transferred every 2 days to successive sterile rearing tubes before dissection. The sex of the flies and the strain of *B. cinerea* were considered. The development features of *B. cinerea* included time of germination, time of hyphal development, and persistence.

Effect of rearing temperature. Groups of male (m) and female (f) flies were reared separately for 1 month at 17°C (groups 1f and 1m), 20°C (groups 2f and 2m), or 20°C for 1 week, then at 17°C (groups 3f, 3m, 4f, and 4m, respectively). Crops of the 4f group were aseptically dissected for in vitro cultivation of the infecting fungus.

RESULTS

Preliminary experiments. Crops dissected from *Drosophila* flies captured in the wild or reared under nonsterile conditions often contained yeasts, bacteria, and fungi in variable quantities. *B. cinerea* conidia developing in *Drosophila* crops were first observed in wild *Drosophila* spp. collected from *B. cinerea*-contaminated grapes and then were transferred to sterile medium. Most *Drosophila* flies collected from vineyards were identified as *D. melanogaster*, less than 10% were *D. simulans*.

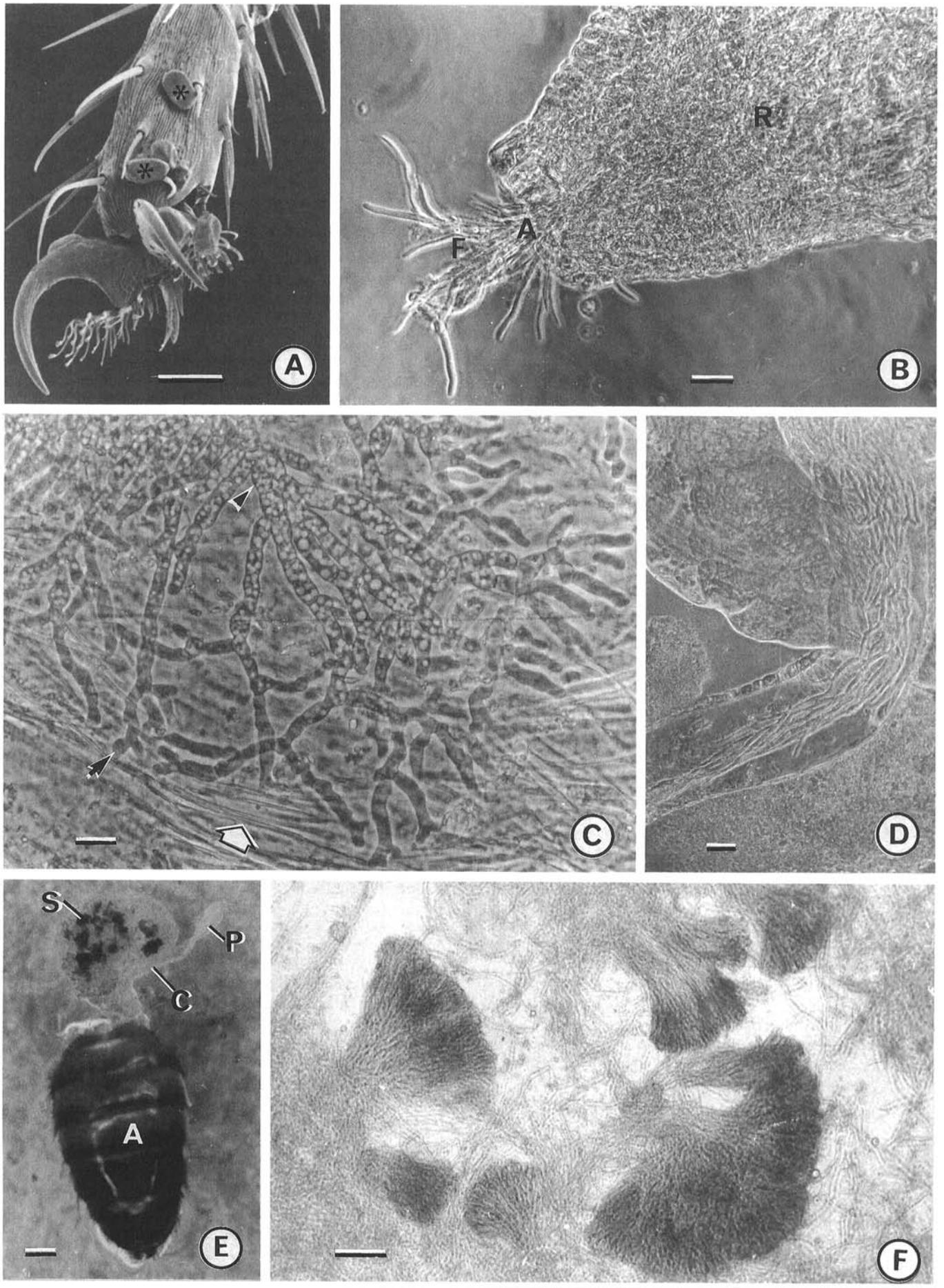
Laboratory experiments. The use of germfree 'Champetières' as experimental flies and defined *B. cinerea* strains allowed standardized laboratory experiments. As a negative control, fungal growth (*B. cinerea* or other fungi) was not observed in rearing bottles of control (uninfected) *Drosophila* flies.

Gross dissemination of *B. cinerea* by *Drosophila*. Short time passages on successive clean media performed with *D. melanogaster* flies (Champetières) contaminated with *B. cinerea* (B1) enabled us to confirm the ability of *Drosophila* flies to transmit *B. cinerea* and to determine how long transmission ability was maintained in flies after the first exposure to the fungus. In female flies, fungal growth was observed in every tube prepared before day 16, whereas the last three tubes representing transfers on days 18 to 22 remained sterile. For male flies, fungal growth was observed in tubes made by transferring flies after 8 to 12, 16, and 24 h and 8 and 10 days. All tubes remained sterile when flies were transferred from days 12 to 22.

External *B. cinerea* transmission. The presence of *B. cinerea* conidia that adhered to the insect cuticle was observed by SEM. Heads and legs were generally the body parts most copiously coated with conidia (Fig. 1A, second tube transfer). The wing and dorsal portions of the body carried very few conidia, even after a single rearing tube change. We did not find conidia borne externally after the fifth tube change.

Conidial transit through the digestive tract. Cultivation of isolated feces resulted in *B. cinerea* (B1 strain) development (7 of 10 assays). Cultivation of feces of uninfected flies remained sterile (10 assays). Microscopic examination immediately after dissection of flies that fed on sporulating fungal cultures showed that rectums were filled with nongerminated conidia. The samples maintained at 20°C for 24 h under a coverslip (five samples) revealed in situ germination of spores from each of the examined rectums (Fig. 1B, strain B1). Rectal examination of uninfected flies gave negative results, with no conidia or hyphae observed.

***B. cinerea* development in *Drosophila* crops.** Conidia were observed for all *B. cinerea* strains examined in more than half of the *Drosophila* crops dissected 2 to 24 h after flies fed on sporulating *B. cinerea* cultures. On the other hand, mycelial filaments that came directly from *B. cinerea* cultures were never detected in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the crops. Swelling and germination of conidia with growth of a germ tube was observed for the B1 and C1 strains of *B. cinerea*. Development of germ tubes into mycelia usually was observed up to 3 days after ingestion of *B. cinerea* conidia (B1 and C1). As usual in fungal colonies, the peripheral area of the mycelium was actively growing (Fig. 1C), which later led to a complete invasion of the crop. In some cases, the mycelium extended out of the *Drosophila* crop to invade the pedunculum that links it to the oesophagus (Fig. 1D). The presence of hyphae in the oesophagus or in the midgut was observed occasionally. We have never observed invasion of the internal organs of the flies or pathogenic effects due to fungal proliferation, similar to the results described by Frobisher (13) for other fungi.

Quantitative study of developmental stages of *B. cinerea* strains in *D. melanogaster* crops. Mycelial development of the S22 fungal strain was not observed in *D. melanogaster* males or females. Fungal development for strains B1 and C1 was observed in 8 of 27 (30%) and in 5 of 32 (16%) *D. melanogaster* females, respectively, but not in males.

A series of experiments was devoted to strain B1. Two days after rearing on sporulating cultures, spores were observed in each of the dissected *Drosophila* crops (five probes). Germinating spores were observed on days 3 and 4 (five probes each). Fungal clusters were observed after 7 days in 7 of 10 females. Surviving insects were reared for 1 month. All of the surviving insects were dissected. Clusters of mycelium were observed in 31 of 74 crops of surviving females (42%) and in 2 of 26 (8%) crops of surviving males.

Effect of rearing temperature. Rearing of flies contaminated with *B. cinerea* (B1) at 17 and 20°C for 1 month resulted in mycelium development from 32 to 46% of fly crops, respectively (groups 1f and 2f, Table 1). No development of mycelium was observed in male crops (groups 1m and 2m, Table 1). Rearing first at 20°C and then at 17°C, resulted in mycelium development in 45, 55, 25, and 40% of surviving flies in groups 3f, 4f, 3m, and 4m, respectively.

Differentiation of peculiar forms of *B. cinerea* (B1) in *D. melanogaster* crops. In three of the nine mycelium-containing crops of the 3f group, we observed a clearly modified mycelium, with differentiation of hardened brown nodules composed of closely packed, short hyphae showing successive dichotomous branching, septation, and darkening (Fig. 1E and F). Repeated experiments (group 4f) yielded brown nodules in 4 of the 11 mycelium-containing crops. Sterile dissection and sterile in vitro cultivation of the 11 crops containing mycelium or the brown nodules (4f group) resulted in *B. cinerea* colony growth.

DISCUSSION

Rearing temperatures higher than 25°C are not optimal for fungal development (2) and lead to sterile mycelium. Lower temperatures are more suitable, with 18°C optimal for germination and 15°C optimal for sclerotium development; *Drosophila* imagos, however, are not fully active below 17°C (21). To make experimental compromises between the different conditions, intermediate temperatures were chosen: the rearing temperature for *Drosophila* was 20°C instead of 25°C (21). Experiments were usually performed at 20°C; long-term experiments (1 month) were performed at 20, 17, and at 20°C for 1 week and then at 17°C for 3 weeks (Table 1).

To avoid possible confusion between fungal spores and whole yeasts usually contained in rearing medium (8) when observing digestive tract contents under a light microscope, a rearing medium containing yeast extract instead of whole yeasts was employed. To allow *B. cinerea* growth, preservative was omitted from the rearing medium for the flies.

The results of the present study with strictly controlled conditions demonstrate the ability of *D. melanogaster* flies to vector *B. cinerea*. The mechanism of *B. cinerea* transmission was precisely studied. *B. cinerea* conidia can be carried both on the fly surface and through intestinal transit, as previously reported in *Rhizopus* transmission by *D. melanogaster* (18) and in *B. cinerea* transmission by grape berry moth larvae (9,10). In addition, long-term *D. melanogaster/B. cinerea* relationships were obtained in the fly crop during the life of the fly. Indeed, *B. cinerea* conidia germinated, developed into mycelium, and differentiated into microsclerotia.

Flies contaminated by transfer to tubes containing sporulating *B. cinerea* were treated by a commonly used method of cleaning external contaminants from insects: successive transfer of flies to sterile tubes. We observed by SEM that after five passages, insects were cleaned of their external contaminants (such as spores), due to both mechanical loss and the cleaning behavior of the flies. The first quick passages (nine passages in 24 h) were sufficient to remove external contaminants. Thus, we conclude that for males the majority of external spores are eliminated in less than 48 h because the tubes collected between days 2 and 6 remained sterile. To explain the late contamination we observed in males (days 8 and 10), we conclude that it has a digestive, rather than an external origin. For females, the difference between the two phases was not apparent, because the tubes were contaminated from days 1 to 16. Both types of transmission, external and digestive, seemed to overlap for female flies.

The reasons for the difference between sexes may be explained by the fact that females ate far more to realize oogenesis, thus the amount of spores that served as inoculum for the digestive proliferation was likely greater, and the intestinal transit was more important. Direct demonstration of the long-term (more than 3 weeks) effective transmission of *B. cinerea* has not been demonstrated previously, due to the difficulty of rearing and keeping an appropriate number of flies in aseptic conditions for such long observation periods. Intestinal transit of conidia and subsequent conidial viability was confirmed by observations of conidia germination in dissected rectums and by in vitro cultivation of conidia. Fungal spores are generally unaffected by passage through the gut,

Fig. 1. Mechanisms of external and internal transmission of *Botrytis cinerea* (B1 strain) conidia by *Drosophila melanogaster* ('Champetières' strain) flies. **A,** External transmission. Scanning electron micrograph of *D. melanogaster* leg after rearing of the fly for 1 h on a sporulating *B. cinerea* culture, followed by two passages of the fly on successive sterile rearing media for 1 h each. Some *B. cinerea* conidia (asterisks) can still be observed. Bar = 10 µm. **B–F,** Transmission through the digestive tract. **B,** Experimental development of hyphae from ingested *B. cinerea* conidia. The terminal portion of the digestive tract (rectum) of *B. cinerea*-reared *D. melanogaster* was dissected 24 h after rearing. The conidia-containing rectum was maintained for 24 h under a coverslip in sterile Drosophila Ringer's solution. Mycelium growing out of the anus was observed, confirming that conidia were able to survive after passage through the digestive tract. F = *B. cinerea* hyphae growing from living conidia. R = rectum. A = anus. Bar = 20 µm. **C–D,** Phase-contrast examination of *B. cinerea* hyphae growing in *D. melanogaster* crops. **C,** *D. melanogaster* crop dissected 4 days after ingesting *B. cinerea* conidia. Conidia present in the crop developed into mycelia. Developing hyphae at the periphery of the colony are indicated by arrows. Older vacuolate stage of *B. cinerea* hyphae is indicated by an arrowhead. Cuticular intima of the crop wall is indicated by an empty arrow. Bar = 10 µm. **D,** *B. cinerea* hyphae invading the crop peduncle. Bar = 20 µm. **E–F,** *D. melanogaster* flies infected for 1 month with *B. cinerea* conidia. Differentiation of *B. cinerea* microsclerotia in the *D. melanogaster* crops. Microsclerotia appeared as small-sized modified fungal hyphae showing successive dichotomous branching, septation, and darkening, as described in *B. cinerea* sclerotia. **E,** Low magnification of dissected crop with brown microsclerotia. Macrophotography. C = crop. P = crop peduncle. A = abdomen. S = microsclerotia. Bar = 200 µm. **F,** Squash of a dissected *D. melanogaster* crop with microsclerotia. Normal light microscopy. The difference between the structure and darkness of fungal hyphae and of modified fungal hyphae composing the microsclerotia are clearly shown. Bar = 50 µm.

as observed by Hasan (16) in Orthoptera transmission of *Colletotrichum graminicola* spores.

We tried to detect *D. melanogaster* bubbling behavior or spore regurgitation as related in Trypetidae (6,17) without success. If this phenomenon occurs, it seems to be of limited importance when compared with transmission by feces deposition.

One of the most significant findings of this work was the observation that spores are often collected in the fly crop, in a particular area of the digestive tract. Consequently, spores potentially could be released later from the crop to the intestine. In the fly crop, at least some of the spores developed in situ into a mycelium that persisted and continued to develop for at least 1 month.

In *B. cinerea*-contaminated females reared for 1 month successively at 20°C and then at 17°C, the observed brown hardened nodules composed of closely packed, short hyphae (Fig. 1E and F) probably represent intermediate stages of sclerotia development. They are very similar to the developing sclerotia described by Coley-Smith (7). We designated them as "microsclerotia," taking into account their particularly small size. In vitro cultivation of fly crop contents confirmed the identity of the *B. cinerea* mycelia and microsclerotia. Thus, *Drosophila* flies once infected become a potential reservoir of rot in three ways: spores, mycelium, and microsclerotia. They may play a role not only in infesting healthy fruit during the production season, but also in overwinter conservation of rots, since *Drosophila* overwinters as an adult (19). It has been shown that *D. melanogaster* can maintain microorganisms through the winter season (Sigma virus [12]).

Important differences were observed between rot strains of the same species. In *B. cinerea*, the B1 strain developed mycelia in 30 to 55% of females but rarely in males. By contrast, the S22 strain did not develop in males or females. The C1 strain presented an intermediate behavior, developing mycelia only in females and at low frequency. The factor responsible for such variability may be related to a difference in the feeding attraction and germinative potential between strains. Such differences may explain the variability we observed in preliminary experiments likely due to the heterogeneity of the *B. cinerea* isolates collected in the wild. Rearing temperature also may play a role in the ability of the different rot strains to develop.

We hypothesize that fly crop infection with *B. cinerea* has no drastic toxic effect on the *Drosophila* flies. However, it could result in a moderately increased mortality of infected flies, explaining the observed decrease in the percentage of mycelium-bearing crops in older flies. The mortality of infected males seemed particularly high (Table 1, 58% of the males died before 1 month).

Drosophila imagos appear to be effective vectors of *B. cinerea* by actively dispersing spores. Using the terminology accepted for *Homoptera* vectors of viruses (15), we can consider *Drosophila* flies as agents of nonpersistent transmission because of their ability to carry spores externally. They also may be considered as agents of semipersistent transmission, because they can release viable spores after passage through the digestive tract. Finally, *Drosophila* can be considered as a potential persistent transmission agent, because the fungus develops in the fly crop and generates resistant forms.

The systematic explorative behavior of *Drosophila* on fruits and grapes at various maturity stages makes the fruit flies a self-guided missile responsible for rot dissemination in vineyards and orchards. The action of *Drosophila* also continues during the conservation and marketing steps. Broadening this discussion into the relative role insects may play in disseminating fungi, gross transmission of fungal pathogens by insects has been demonstrated in beetles, thrips, and flies (15,20). Agrios (1) recorded insect involvement in the transmission of fungal pathogens. Hasan (16) and Fermaud and Le Menn (11) demonstrated that ingested fungal spores were recovered in viable condition from feces of locusts and grape berry moth larvae, respectively. As reported by Hasan (16), few studies have demonstrated that diseases other than viruses are carried internally. Moreover, the mechanism of internal transmission of fungi by insects is a subject that is not well described or studied. Our own experience, the present paper, and research on *Ceratitis* by Cayol et al. (6) allow us to agree with Agrios (1) on the need for studies on the importance of insects as bunch rot vectors.

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TABLE 1. Influence of rearing temperature of *Drosophila melanogaster* ('Champetières' strain) flies on the development of the fungus *Botrytis cinerea* (B1 strain) in the fly crop[a]

	Females							Males					
Temp. (C)	Group no.	Total no.	No. dead[b]	No. diss.	No. mycel.	Mycel./ diss. (%)[c]	Scler.[d]	Group no.	Total no.	No. dead[b]	No. diss.	No. mycel.	Mycel./ diss. (%)[c]
17[e]	1f	38	10	28	9	32	0	1m	23	11	12	0	0
20[e]	2f	33	9	24	11	46	0	2m	23	15	18	0	0
20/17[f]	3f	20	0	20	9	45	3	3m	20	12	8	2	25
20/17[f]	4f	25	5	20	11	55	4	4m	25	15	10	4	40

[a] Flies were reared on sporulating *B. cinerea* cultures for 12 h and then were transferred every 2 days in sterile rearing tubes.

[b] After 1 month, mortality was higher in male than in female flies, as usual in *D. melanogaster* rearing. The surviving flies were dissected (diss.).

[c] *B. cinerea* mycelium (Mycel.) was observed in the fly crops of 32 to 55% of the surviving females and in the surviving males from 0% (rearing temperature = 17 or 20°C) to 40% (rearing temperature = 20°C for 1 week and 17°C thereafter).

[d] Microsclerotia (Scler.) were observed in 3 of the 9 and 4 of the 11 mycelium-containing female fly crops of groups 3f and 4f, respectively, of the 20/17°C experiments.

[e] Groups 1 (1f = females, 1m = males) and 2 (2f and 2m) were reared for 1 month at 17 and 20°C, respectively.

[f] Groups 3 (3f and 3m) and 4 (4f and 4m) were reared at 20°C for 1 week then at 17°C.

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