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Quantitative postharvest contamination and transmission of *Penicillium expansum* (Link) conidia to nectarine and pear fruit by *Drosophila melanogaster* (Meig.) adults

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Abstract

This research demonstrated the possibility of conidial transmission of *Penicillium expansum* by the adult flies of *Drosophila melanogaster* to mature, sound nectarine and pear fruit. This transmission was accomplished by inserting the fungal conidia adhering either to mouthparts of the contaminated flies or to their abdominal tip into mature, sound nectarine and pear fruit, while making punctures in the fruit skin either for feeding or for oviposition. Accordingly, the mean number of typical *P. expansum* lesions that appeared due to this transmission per one nectarine or pear fruit subjected to contaminated flies was 4.7 and 2.5, respectively. Also, the mean diameter of these typical lesions was 5.3 and 3.2 mm on the same types of fruit, respectively. When the eggs laid by the contaminated females of *D. melanogaster* were left to develop until adult fly emergence, the mean number of the flies that emerged per fruit at the end of the life cycle was 48.3 and 24.3 on nectarine and pear fruit, respectively. Also, the mean life cycle duration for the emerged flies was 24.3 and 28.7 days on the same types of fruit, respectively. Moreover, viability of the pathogen conidia that either adhered externally to the various body parts of the contaminated flies or were introduced into their bodies was tested by plating the conidia onto oatmeal agar plates amended with chloramphenicol, following the release of the contaminated flies onto plates or the spread of their ground suspension in saline solution onto the same type of plates. The mean number of typical *P. expansum* colonies that appeared per plate was 5.3 for external contamination of the flies and 2.4 for internal contamination. The conidia of *P. expansum* adhering to the various body parts of contaminated flies were first localized on these parts and then photographed under the light microscope after they have been correctly identified. Overall results indicate the possibility of *P. expansum* conidial transmission by *D. melanogaster* adults into sound, mature nectarine and pear fruit through their feeding and oviposition punctures.

Keywords: Blue mold; Fruit flies; *Penicillium expansum*; *Drosophila melanogaster*; Conidial transmission; External contamination; Internal contamination

1. Introduction

Penicillium expansum is a major pathogen on pome fruit, although *Monilinia* spp., *Botrytis cinerea* and *Rhizopus stolonifer* are the major pathogens on stone fruit. *P. expansum* is the causative organism of blue mold that infects many types of pome and stone fruit at the postharvest stage (Palazon et al., 1984) (Fig. 1A and B). Control of the disease, for example on apples, is critical to the apple industry because infection with *P. expansum*, especially in the core rot phase of the disease, is also accompanied by production of the mycotoxin, patulin, in the rotten tissues of infected apples (Brain, 1956; Harwig, 1973). Apple juice processed from rotten apples usually contains high levels of patulin since thermal processing appears to cause only moderate reduction in the mycotoxin levels, thus its presence in apple juice will survive the pasteurization processes (Harison, 1989).

Fruit flies, especially *Drosophila melanogaster* (Fig. 1C–E) and *Ceratitis capitata*, are cosmopolitan insect pests of many species of commercial and wild fruit. These flies have been proven to be potential vectors of many bacterial pathogens, which could be transmitted by these insects to fruit and then to humans. For example, transmission of

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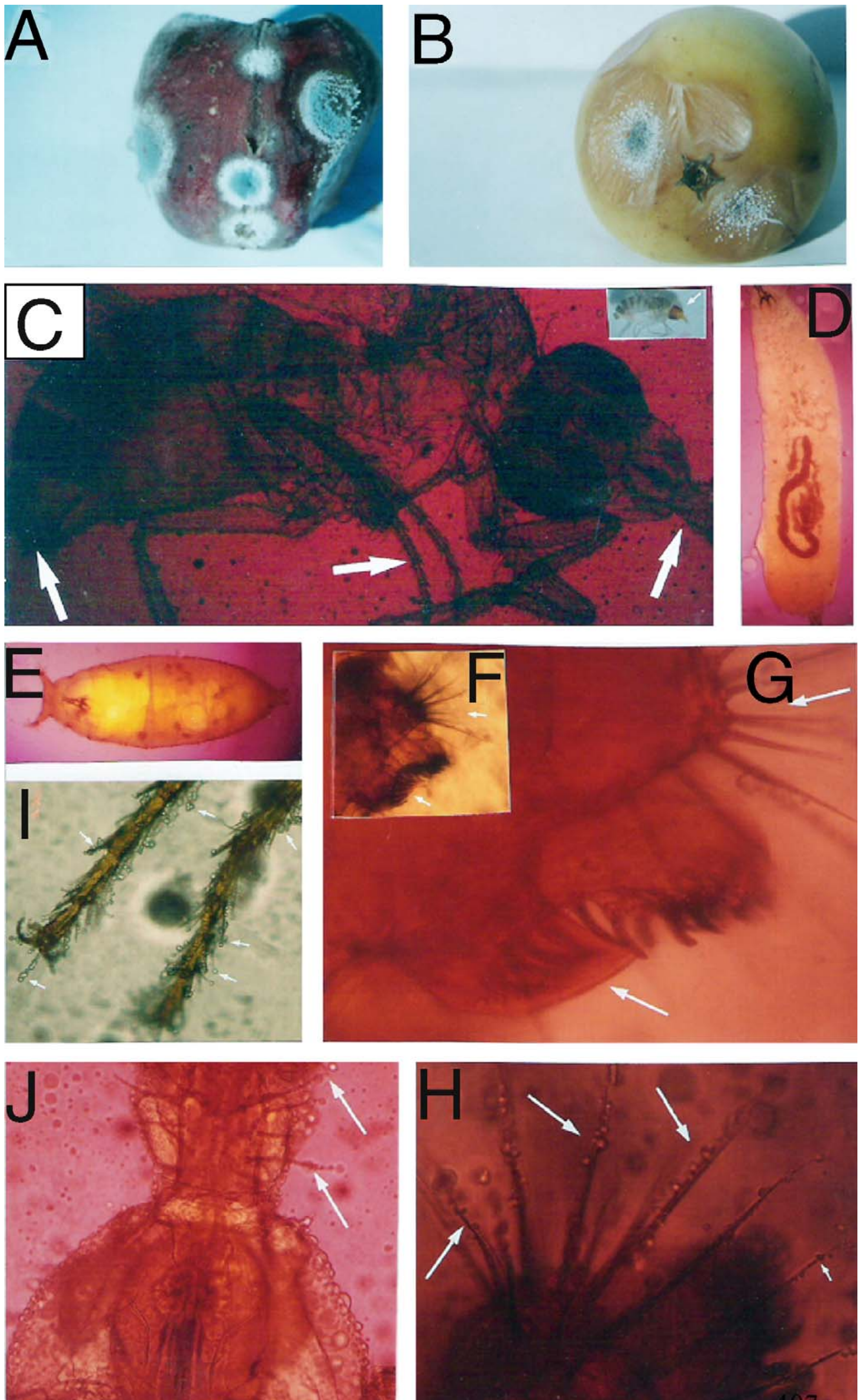
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Escherichia coli to mature fruit by *C. capitata* (Janisiewicz et al., 1999; Sela et al., 2005); Drosophila C virus by *D. melanogaster* (serves as a host) where viral particles were detected in the faeces of a virus-infected-female cadaver (Lantie-Harivel and Thomas-Orillard, 1990; Gomariz-Zibler et al., 1998); *Wolbachia pipentis* (intracellular bacterial pathogen) by *D. melanogaster* and *D. simulans* (Mercot and Poinsot, 1998; McGraw and O'Neill, 1999; Olsen et al., 2001); and the plant parasitic nematode, *Howardula aoronymphium* by *D. melanogaster* (Jaenike, 2000).

Transmission of *P. expansum* conidia from infected tissues might occur by direct contact between diseased and healthy fruit when stored in the same container or in the same warehouse. For transmission over longer distances, human or insect vectors are needed. To the best of our knowledge, no reports on contamination and transmission of *P. expansum* conidia to sound fruit by *D. melanogaster* have been found. Therefore, the objectives of the present research were: (i) to show the possibility of *P. expansum* conidial transmission by contaminated flies of *D. melanogaster*; (ii) to describe the mode of conidial transmission by the flies when the possibility of transmission is shown and (iii) to identify then localize and photograph the conidia transmitted by the flies using light microscopy.

2. Materials and methods

2.1. Strain of D. melanogaster used in bioassays

Pupae of the fruit flies (*D. melanogaster*, strain DM1) were obtained from infested nectarine fruit. These pupae were incubated at 20 ± 2 °C and 16 h of illumination per day in sterile plastic cans (9 cm diameter × 8 cm deep) covered with thin transparent plastic film until the adult flies emerged. Newly emerged male and female adult flies were released onto rearing synthetic medium (Tegosept-M) poured aseptically in 50 ml cotton-plugged rearing bottles. F1 male and female adults emerged during rearing were used in conducting bioassays since these adults were free from any contamination.

Tegosept-M used for rearing the insects contained *p*-hydroxybenzoate as a mold inhibitor (Carolina Biological Supply Company, 1987). It was prepared by adding 15 g of agar and 1.5 g of *p*-hydroxybenzoate to 500 ml boiling water. One hundred and thirty milliliter of sulfur-free molasses were then added to the above-mentioned solution and brought again to boiling point. One hundred gram of dry yellow cornmeal were mixed with 250 ml of cold water then poured into the above-mentioned boiling solution, then cooked for a few minutes. Small quantities, 2.5 cm deep, of the cooked medium were poured in each 50 ml sterile rearing bottle plugged with cotton, then left until they become solid. There was no need for sterilization of the medium (Carolina Biological Supply Company, 1987).

2.2. Strain of P. expansum used in experiments

The *P. expansum* strain PE3, isolated from infected apple fruit with blue mold disease, was used in the bioassays. It was first isolated on oatmeal agar medium then kept as a pure culture on the same medium at 4 °C for being used in subculturing of the fungus during infection of fruit with the pathogen.

2.3. Infection of nectarine fruit with P. expansum to contaminate adults of D. melanogaster

Healthy mature nectarine fruit (*Prunus persicae* var. *nectarina*, cv. Silver load) were inoculated with 25 μl-droplets of *P. expansum*-conidial suspension (strain PE3; concentration 3.2×10^5 conidia/ml) after being superficially disinfected with 0.025% sodium hypochlorite then rinsed with sterile distilled water and wounded. The inoculated fruit were then incubated in sterile plastic cans (9 cm diameter × 8 cm deep) covered with thin transparent plastic film at 20 ± 2 °C and 16 h of illumination per day until the appearance of typical blue mold lesions on the fruit surface. The resulting blue-molded fruit (1 week after harvest of mature, sound fruit) were then used for contamination of *D. melanogaster*-adult flies with the conidia of the pathogen by releasing newly emerged males and females of F1 adults obtained from the rearing bottles (Section 2.1) onto blue-mold contaminated fruit for 72 h. Contaminated flies were then used for bioassays of conidial transmission of the pathogen.

2.4. Bioassays of conidial transmission of P. expansum by contaminated D. melanogaster adult flies to healthy fruit

Three males and females of contaminated flies of *D. melanogaster* by *P. expansum* (Section 2.3) were introduced into each tightly closed plastic can (9 cm diameter × 8 cm deep) containing one healthy mature and superficially disinfected nectarine or pear fruit (1 day after harvest of mature,

Fig. 1. Modality of conidial transmission of *Penicillium expansum* to mature healthy nectarine and pear fruit by adult flies of *Drosophila melanogaster*. A and B, typical lesions of *P. expansum* on nectarine and pear fruit, respectively, showing blue-colored conidial layer of the pathogen on the lesion surface. C, general view of adult fly of *D. melanogaster* (small and large, 40×, views), arrows show the body parts that may be involved in the pathogen transmission. D, general view (40×) of *D. melanogaster* larva (last larval instar). E, general view (40×) of *D. melanogaster* puparium prior to adult emergence. F and G, tip of abdomen of *D. melanogaster* adult (100×) showing long hairs and nearby ovipositor to which *P. expansum* conidia may adhere (arrows). H, enlarged view (400×) of F and G showing typical conidia of *P. expansum* adhered to the long hairs projecting from tip of abdomen of *D. melanogaster* (arrows) adjacent to the ovipositor. I, forelegs of *D. melanogaster* adult showing typical adhered *P. expansum* conidia (100×) to the hairs projecting from its tarsal segments. J, enlarged view (400×) of *D. melanogaster* proboscis (mouthparts) showing typical *P. expansum* conidia (arrows) adhered to the hairs projecting from its anterior part.

sound fruit). The introduced flies were left in the cans for 72 h before being removed. This time period was sufficient for flies to feed and to lay eggs on the fruit or to contaminate them. Ten replicates representing 10 plastic cans were used. Each can contained one fruit of each type (nectarine or pear). The control treatment comprised cans with pear or nectarine fruit subjected to non-contaminated flies that were obtained from rearing bottles for comparison. All cans with fruit were then incubated at 20 ± 2 °C and 16 h of illumination per day until the appearance of typical *P. expansum* lesions on the treated fruit and until the emergence of F2 adult flies as a result of egg-laying by the introduced F1 adult flies on treated fruit. The diameter and number of typical *P. expansum* lesions per fruit, in addition to the number of emerged adult flies per can and duration of the life cycle due to this emergence were determined and measured in the bioassays.

2.5. Viability of P. expansum conidia transmitted by contaminated D. melanogaster-adult flies

To determine the level of external contamination of *D. melanogaster* flies by *P. expansum* conidia, one adult fly contaminated with the pathogen (Section 2.3) was introduced into each plate containing oatmeal agar + chloramphenicol as a selective medium, for 7 h to contaminate the culture medium with *P. expansum* conidia. This time period was sufficient for making contact between the fly body parts and the culture medium surface. Each plate contained 12 ml of autoclaved oatmeal agar medium poured under aseptic conditions after adding chloramphenicol (250 mg/l) as an effective antibiotic to bacterial growth. The control treatment comprised plates with culture medium + chloramphenicol subjected to non-contaminated flies that were obtained from the rearing bottles for the purpose of comparison. To determine the level of internal contamination of *D. melanogaster* flies by *P. expansum* conidia, the contaminated flies were placed in a freezer for approximately 10 min and then each fly was put in a test tube with 5 ml of 70% ethanol for 1 min to be superficially sterilized. It was then blotted on sterile filter paper, transferred to a test tube with 5 ml of sterile distilled water, blotted on filter paper again and ground with a mortar and pestle in 5 ml of NaCl solution (0.025% w/w). The suspension was plated on oatmeal agar medium amended with chloramphenicol at a rate of 100 μl per plate. All plates were then incubated at 20 ± 2 °C and 16 h of illumination per day until appearance of the typical *P. expansum* colonies on the surface of culture medium in each plate. The number of typical *P. expansum* colonies per plate and the time needed for this appearance on the plate surface were determined.

2.6. Statistical analyses

The mean number and mean diameter of typical *P. expansum* lesions appearing on the nectarine and pear fruit exposed to contaminated flies of *D. melanogaster* were calculated and statistially analyzed using ANOVA and Duncan's multiple range test (DMRT). Moreover, the mean number of adult flies (F2 adults) emerged at the end of the life cycle due to the introduction of F1 adults and the mean life cycle duration were calculated and then analyzed using the same statistical methods.

3. Results

3.1. Transmission of P. expansum conidia to healthy mature fruit by contaminated adult flies of D. melanogaster

Releasing contaminated adult flies of *D. melanogaster* with *P. expansum* conidia onto healthy mature nectarine and pear fruit for 72 h resulted in infection of the fruit with the typical *P. expansum* lesions. The mean number of these typical lesions that appeared on the fruit surface 6 days after incubation of the fruit was 4.7 on nectarine fruit and 2.5 on pear fruit (Table 1). Significant differences ($P < 0.05$) between the two means were obtained.

However, no symptoms of infection with typical *P. expansum* lesions were observed on the nectarine and pear fruit subjected to non-contaminated *D. melanogaster* adult flies. Moreover, the mean diameter of typical *P. expansum* lesions that were observed on the fruit surface 6 days after incubation of the treated fruit was 5.3 mm on nectarine fruit and 3.2 mm on pear fruit. Significant differences ($P < 0.05$) between the two means were also obtained. These results indicate that *D. melanogaster* adults are efficient vectors for transmission of *P. expansum* conidia to the tested fruit (nectarine and pear fruit) at the postharvest stage. When the fruit of both types that were subjected to contaminated adults of *D. melanogaster* were left incubating until the emergence of F2 adults, the mean number of adult flies per fruit that emerged at the end of life cycle was 48.3 on nectarine fruit and 24.3 on pear fruit. Significant differences (at $P < 0.05$) between the two means were obtained. Similar significant results were obtained in the mean duration of the *D. melanogaster* life cycle on the same types of fruit. Therefore, the mean life cycle duration of *D. melanogaster* on pear fruit was significantly longer than that on nectarine fruit (28.7 and 24.3 days, respectively). These results confirm the capacity of *D. melanogaster* adults for transmission of *P. expansum* conidia to mature pear and nectarine fruit. The adult flies preferred nectarine fruit to pear fruit for this transmission. No significant differences were obtained between the mean number of adult flies emerged at the end of the life cycle on nectarine fruit subjected to contaminated and non-contaminated adults of *D. melanogaster* (48.3 and 50.2 adults, respectively). The same results were obtained when pear fruits were subjected to contaminated and non-contaminated adults of *D. melanogaster* (24.3 and 26.1 adults, respectively) (Table 1). Therefore, the transmission of *P. expansum* to nectarine fruit was higher than that to pear fruit, and that the

Table 1
Development of infection with blue mold (*Penicillium expansum*, strain PE3) on mature pear and nectarine fruit when subjected to contaminated adults of *Drosophila melanogaster* (strain DM1) after incubation of subjected fruit at 20 ± 2 °C and 16 h of illumination per day under humid conditions

Type of mature fruit subjected to adult flies of *D. melanogaster*	Number of typical *P. expansum* lesions per fruit 6 days after incubation of subjected fruit[a]		Diameter of typical *P. expansum* lesions (in mm) appeared on the subjected fruit 6 days after incubation[a]		Duration of life cycle of *D. melanogaster* (in days) indicated by emergence of adult flies per fruit[a]		Number of adult flies of *D. melanogaster* emerged at the end of life cycle per fruit[a]	
	Range	Mean	Range	Mean	Range	Mean	Range	Mean
Nectarine fruit subjected to contaminated flies[b]	4–7	4.7 c[c]	3–8	5.3 c[c]	21–26	24.3 a[c]	31–69	48.3 b[c]
Nectarine fruit subjected to non-contaminated flies[d]	0	0 a	0	0 a	20–25	24.1 a	35–74	50.2 b
Pear fruit subjected to contaminated flies[b]	2–5	2.5 b	2–5	3.2 b	25–31	28.7 b	14–41	24.3 a
Pear fruit subjected to non-contaminated flies[d]	0	0 a	0	0 a	25–30	27.8[d] b	18–42	26.1 a

[a] Ten replicates representing 10 fruit of each type incubated in plastic cans (one fruit/can).
[b] Each fruit type was subjected to three males and three females of contaminated flies.
[c] Means within each column followed by different letters are significantly different at $P < 0.05$.
[d] Non-contaminated male and female adult flies were obtained from insect rearing bottles.

duration of life cycle of *D. melanogaster* was not different whether the fruit were infected with the pathogen or not.

3.2. *Viability of P. expansum conidia transmitted by contaminated adult flies of D. melanogaster*

Releasing contaminated adult flies of *D. melanogaster* with *P. expansum* conidia onto plates containing oatmeal agar medium amended with chloramphenicol for 72 h resulted in the appearance of typical *P. expansum* colonies on the surface of the treated medium in the plates. Therefore, the mean number of observed typical *P. expansum* colonies per plate was 5.3, and the mean time required for the appearance of such typical colonies was 5.1 days (Table 2). However, no typical colonies of *P. expansum* were observed on the medium surface subjected to non-contaminated adult flies of *D. melanogaster*. Moreover, plating the suspension with the mixture derived from grinding the internally contaminated, but superficially disinfected adult flies, in saline solution, resulted in the appearance of typical *P. expansum* colonies on the surface of the medium. The mean number of typical *P. expansum* colonies per plate was 2.4 and the mean time required for appearance of such colonies was 5.8 days (Table 2). These results show the possibility of *P. expansum* conidial transmission by *D. melanogaster* adults externally and internally.

3.3. *Mode of transmission of P. expansum conidia by contaminated adults of D. melanogaster*

The conidial transmission of *P. expansum* was shown in the present research by two ways: (i) conidia adhered either to hairs projecting from the tip of fly abdomen near the ovipositor (Fig. 1F–H) or hairs projecting from the anterior part of the fly proboscis (Fig. 1J) were inserted by the fly under the skin of the fruit through their oviposition and/or feeding punctures, (ii) conidia adhered to other parts of the fly body especially to the legs (Fig. 1I) were inserted under the fruit skin through microwounds or natural openings present on the fruit surface. The growth of typical colonies of *P. expansum*

Table 2
Growth of *P. expansum* (strain PE3) on oatmeal agar (OMA) plates + chloramphenicol after releasing contaminated flies of *D. melanogaster* (strain DM1) onto plates or a plating suspension of ground contaminated flies in saline solution and then incubation of plates at 20 ± 2 °C and 16 h of illumination per day until appearance of typical *P. expansum* colonies

OMA plates subjected to *D. melanogaster* adults or plated with suspension of ground contaminated flies	Number of typical blue mold colonies per OMA plate appeared on the plate surface[a]		Time (in days) needed for the appearance of typical blue mold colonies per one OMA plate[a]	
	Range	Mean	Range	Mean
Plates subjected to contaminated flies[b]	3–10	5.3	4–6	5.1
Plates subjected to non-contaminated flies[c]	0	0	[d]	[d]
Plates plated with suspension of ground contaminated flies in saline solution	2–3	2.4	4–7	5.8

[a] Ten replicates representing 10 plates with pure culture medium of OMA + chloramphenicol.
[b] One contaminated adult of *D. melanogaster* were released onto OMA + chloramphenicol plates for 72 h.
[c] One non-contaminated adult of *D. melanogaster* obtained from insect rearing bottles was used.
[d] The time needed for appearance of typical *P. expansum* colonies on medium plates was extended to 10 days but without any appearance of pathogen typical colonies.

on the plate surface of oatmeal agar amended with chloramphenicol as a result of touching contaminated flies with the medium surface indicated that conidia adherng to various fly body parts were viable and may germinate if touching the medium surface (Table 2), or may cause infection if transmitted by the fly to mature healthy fruit.

4. Discussion

In the present research, two types of fruit were chosen as hosts for *D. melanogaster* due to their host preference across different types of fruit (Elzinga, 1997). This host preference explains the presence of significant differences between the number and diameter of typical *P. expansum* lesions observed on nectarine fruit as a preferred host for the flies and pear fruit as a less preferred host following transmission of *P. expansum* conidia by these flies to the two types of fruit. The occurrence of these typical lesions on both types of fruit clearly indicates the possibility of pathogen transmission to these fruit, but to a varying degree according to the insect preference.

A significant number of typical *P. expansum* lesions on healthy mature pear and nectarine fruit were observed in the present study as a result of successful transmission of *P. expansum* conidia by *D. melanogaster* adults. This indicates that the fly adults are potential vectors for *P. expansum* conidial transmission to fruit. This was shown where female flies laid their eggs in the sound fruit by puncturing the fruit skin with their sharp ovipositors and then inserting the eggs into the puncturing wounds, with typical *P. expansum* lesions appearing around their oviposition or feeding punctures. What has been shown for other fruit flies such as *C. capitata* is that they can transmit bacterial pathogens, especially *E. coli*, to rotten fruit to secure nutrients for their offspring (Janisiewicz et al., 1999; Sela et al., 2005). The potential capacity of *D. melanogaster* for transmitting the disease-causing organisms was reported for *Mucor piriformis* on rotten peach fruit (Michailides and Spotts, 1990), *Geotrichum* and *Rhizopus* species on rotten tomato fruit (Butler and Baker, 1963), in addition to the transmission of *Wolbachia pipentis* and Drosophila C virus to other arthropod species (Gomariz-Zibler et al., 1998; Mercot and Poinsot, 1998; McGraw and O'Neill, 1999; Olsen et al., 2001), but without any descriptions of the mechanisms of pathogen transmission. However, no reports have previously been found on the transmission of *P. expansum* by *D. melanogaster* or by any other type of fruit fly.

The present research has shown that *D. melanogaster* adults contaminated with *P. expansum* conidia can transmit the pathogen to healthy or sound fruit. It describes the mechanisms of conidial transmission by this insect to sound fruit, and until the present results, it has not been known whether *P. expansum* requires a wound or bruise for infection to proceed. *D. melanogaster*, in the present study, both makes this wound and inoculates. Also, until the present results, *Drosophila* spp. have not been thought to attack sound fruit, but only over-ripe, rotting, or damaged fruit. The present work demonstrates insect attack on sound fruit. In the light of that, these observations are important for the fruit industry, with the assumption that it should only market sound fruit? Internal contamination of *D. melanogaster* adults with *P. expansum* conidia and then their transmission was also demonstrated here, but the location of these conidia inside the fly body was not specified exactly. Therefore, additional research on localizing and photographing these conidia inside the fly is recommended. For this purpose, molecular techniques such as adding green fluorescent protein (GFP) tagged to *P. expansum* conidia to the fly visiting contaminated fruit, then applying fluorescence microscopy are recommended for localizing the conidia inside the fly body. The use of these techniques and fluorescence microscopy by other investigators has revealed the presence of *E. coli* in the pseudotrachia of the labellum edge of a contaminated *C. capitata* proboscis (Sela et al., 2005).

In conclusion, the present research adds something new to our knowledge on postharvest fruit decay by *P. expansum* since the results obtained on the potential transmission of the pathogen by *D. melanogaster* to sound fruit and their high number of offspring produced per fruit, especially on nectarine fruit, confirm the necessity of controlling these flies to avoid the double damage caused by the transmitted pathogen by the flies themselves and their larval feeding inside the attacked fruit. Although the present study is the first one that deals with the quantitative contamination and transmission of blue mold disease to sound fruit at the postharvest stage by *D. melanogaster* adults, further research is recommended to be conducted to localize the conidia of the pathogen that enters the fly body during contamination with the pathogen. In addition, it would be very significant if it could be shown that *D. melanogaster* are capable of infecting sound fruit with disease agents in order to hasten decomposition and provide more food for offspring. That is an interesting hypothesis, but needs to be supported by scientific evidence.

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