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Characterizing members of the *Cladosporium cladosporioides* species complex as fruit rot pathogens of red raspberries in the mid-Atlantic and co-occurrence with *Drosophila suzukii* (spotted wing drosophila)

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Abstract Fruit rot, primarily caused by *Botrytis cinerea*, is a major driver of pre- and post-harvest yield losses in fall red raspberries in the Mid-Atlantic. Recent observations indicate that *Cladosporium* fruit rot may also reduce yields. In a two-year survey of two Maryland farms, 1–30% of fall-bearing red raspberry fruit exhibited *Cladosporium* fruit rot (CFR) symptoms pre-harvest and symptoms developed post-harvest in 16% of fruit harvested when under-ripe and 51% of fruit harvested red ripe. *Cladosporium cladosporioides* and *C. pseudocladosporioides* were identified based on BLAST analysis of TEF-1α and actin gene regions, but determinations against voucher specimen sequences were not always congruent with phylogenetic analyses; sequences from many isolates matched *C. anthropophilum* voucher sequences still labeled as *C. cladosporioides*. Isolates of all three species caused fruit rot on non-wounded drupes (33–66%) and incidence increased when drupes were wounded (50–100%) ($P < 0.05$). In the field, 21–33% of CFR-affected fruit were infested with *D. suzukii* larvae; *Cladosporium* propagules were recovered from frass of 25–71% of larvae and determined to be *C. cladosporioides* or *C. pseudocladosporioides*, conspecific with fruit-derived isolates. Further, frass-derived isolates of both species initiated rot, with similar incidence to raspberry-derived isolates ($P > 0.05$). This first description of *Cladosporium* fruit rot of red raspberry in the Mid-Atlantic provides an expansive perspective on CFR pre- and post-harvest biology as well as pathogen diversity. Several lines of evidence point to linkages between CFR and *D. suzukii* epidemiology which require further exploration.

Keywords *Cladosporium* fruit rot · *Cladosporium* cladosporioides species complex · *Cladosporium pseudocladosporioides, Cladosporium anthropophilum,* insect frugivore

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Raspberries (*Rubus idaeus* L.) are a small but significant contributor to the Mid-Atlantic horticultural crop industry as a local market and agrotourism crop. Fall red raspberry production is increasing in popularity for fall

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pick-your-own, since it provides a reliable alternative to apples (the primary fall fruit crop) in years with delayed or poor apple yields. Fungal fruit rots are one of the most significant drivers of raspberry yield losses in the region, and a major barrier to organic production. Fungal infections established in the field can lead to fruit rot pre-harvest or can remain latent, leading to post-harvest fruit rot, significantly reducing shelf life.

Botrytis cinerea (the cause of Botrytis fruit rot or grey mold) has been the only known pre-harvest fruit rot pathogen of raspberries in the Mid-Atlantic. However, in 2015, raspberry growers began observing diseased berries that lacked the characteristic signs and symptoms of *B. cinerea* (Ellis et al. 1991). Mycelium was more matted and olive colored rather than grey; as the disease advanced, the fruit blacked, similar to severe Botrytis fruit rot (Fig. 1). In that year, some affected growers in Southern Maryland estimated losing up to 90% of marketable fruit. In preliminary assessments we recovered *Cladosporium* isolates from symptomatic fruit, indicating the disease was *Cladosporium* fruit rot (CFR), which has been reported as a post-harvest pathogen in other regions of the US, but has not been described as a pre-harvest pathogen, nor is it known to occur in the Mid-Atlantic (Ellis et al. 1991).

In preliminary field surveys in 2015 we observed *Drosophila suzukii* larvae in some CFR-infested berries. Although insect-facilitation of phytopathogenic *Cladosporium* is not well described in the literature, looking beyond agricultural systems, there is evidence that several *Cladosporium* species have a symbiotic relationship with diverse arthropods across a wide range of groups (Acari, Araneae, Coleoptera, Collembolla, Diptera, Hemiptera, Hymenoptera, Hymenopetera: Formicidae, Lepidoptera, Neuroptera, Opiliones, Psocoptera, Thysanura and Trichoptera) (Grief and Currah 2007; Trovao et al. 2013). Red raspberries are a particularly attractive host for *Drosophila suzukii* (spotted wing drosophila), a recently introduced pest in the region (Bellamy et al. 2013; Burrack et al. 2013). Females use their saw-like ovipositor to lay eggs in ripe or ripening fruit, instead of in wounded or overripe fruit like other drosophilids (Walsh et al. 2011); wounds created in ripening and ripe fruit may serve as infection courts for fruit rot pathogens. It therefore seemed plausible that *Cladosporium* fruit rot epidemiology may be influenced by *D. suzukii*. Despite a surge of research efforts on this insect pest in recent years, there very are few studies connecting epidemiology of *D. suzukii* and fruit rots. One study in grapes concluded that fungal fruit rot development was independent from fly infestation (Rombaut et al. 2017), and other studies suggest that *D. suzukii* may be capable of vectoring the spoilage microbes that cause sour rot in grapes (Ioriatti et al. 2017; Rombaut et al. 2017). Initially, exploratory studies are needed to establish a connection between *Cladosporium* fruit rot and *D. suzukii*, providing foundational information for downstream epidemiological and management studies.

The primary aim of this study was to elucidate disease etiology of the recently detected putative *Cladosporium* fruit rot (CFR) in Mid-Atlantic red

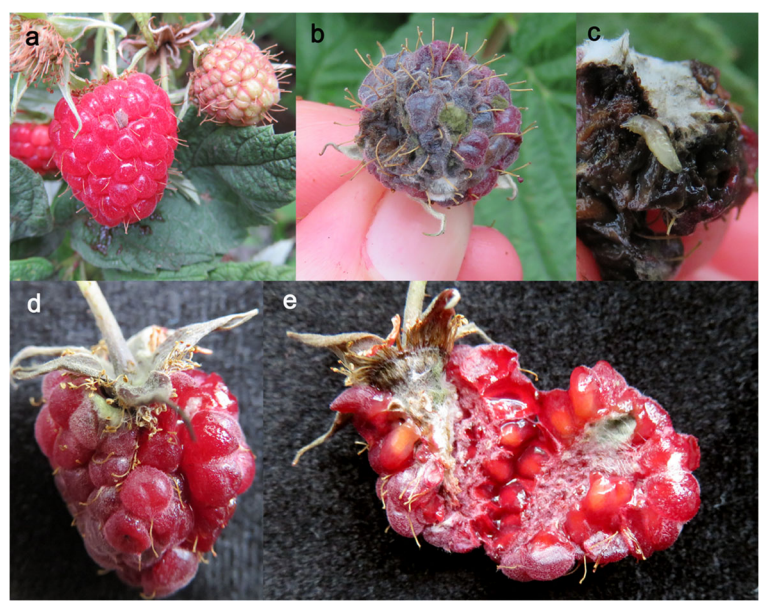


Fig. 1 *Cladosporium* fruit rot signs and symptoms in the field, including (**a**) minor fruit rot, wherein greenish-white to dark green mycelium was observed on one to three drupes/berry, (**b**) severe fruit rot in which mycelium expanded to cover a majority of the berry surface and was associated with tissue necrosis, and showing (**c**) *D. suzukii* larvae inhabiting CFR-affected fruit. In some cases, there were no obvious external symptoms (**d**), but a grey green mycelial pad was present in the inner fruit core, below the fruit cap (**e**)

raspberry fields; the secondary aim was to determine whether *D. suzukii* could be associated with *Cladosporium* species causing CFR. To this end, this study had three integrated objectives to: (1) conduct field surveys to evaluate CFR incidence pre- and post-harvest (considering ripeness of fruit when harvested), and evaluate incidence of CFR-*Drosophila suzukii* co-occurrence in red raspberries; (2) characterize *Cladosporium* species associated with diseased raspberries and with frass of *D. suzukii* larvae collected from the field, phylogenetically verifying interpretation of BLAST results for both raspberry and larval frass isolates; (3) evaluate the ability of *Cladosporium* species recovered from diseased raspberries to cause fruit rot with and without wounding and, secondarily, determine whether *Cladosporium* species from larvae frass could also cause fruit rot. Studies of association between *Cladosporium* species and *D. suzukii* were complimented with a concurrent sister study of whole fungal communities associated with *D. suzukii* frass (Lewis et al. 2018).

Methods

Field surveys: *Cladosporium* fruit rot (CFR) surveys in fall red raspberry fields and presence of *Drosophila suzukii* in CFR-affected fruit

Pre-harvest CFR incidence Pre-harvest fruit rot incidence was evaluated in the fall, on Aug 28, 2015 in Germantown, MD (var. Caroline) and Woodbine, MD (var. Jaclyn), and on Sept 28, 2016 in Germantown, MD (same farm as 2015, var. Himbo Top). Dates and sites were chosen based on grower reports of fruit rot and availability of the site; repeat surveys of each site were not conducted in 2016 since 2015 studies indicated that a single, late season assessment was sufficient to capture incidence. At both sites, raspberries were grown in approximately 1.25 m wire-trained hedges and pests were managed using conventional synthetic pesticides throughout the production season (application every 7–14 days; fungicides in rotation included Captan, Cyprodonil, Fenhexamide, Iprodoine, Pyraclostrobin and Boscalid).

CFR incidence was evaluated in three rows at each site (approximately 30 m/row). The fruit were evaluated for signs and symptoms of *Cladosporium* fruit rot (as detailed above and in Fig. 1a, b, d, e). Surveys attempted to account for variability in CFR distribution within the canopy by examining fruit in each of four canopy locations: (1) lower fruit in the canopy exterior (~10–50 cm from canopy floor); (2) topmost fruit of the canopy exterior (~100–150 cm from canopy floor); (3) topmost fruit of the canopy center (~30–60 cm from the edge); and (4) lower fruit of the canopy interior (~30–60 cm from the canopy floor). Ten fruit were randomly examined in each location, for a total of 40 fruit per row, 120 fruit / field and 360 fruit across the two-year survey. Confirmation as CFR was subsequently analyzed as described below.

Post-harvest CFR incidence Post-harvest CFR incidence was evaluated at one site (Woodbine—selected at random) in 2015; to evaluate whether maturity of fruit at harvest influenced disease development, we examined both ripe (red ripe) and unripe (pink) berries. The experiment was arranged in a completely randomized design, with six replicates per treatment and five berries per replicate. The experiment was conducted twice, using berries collected on September 15 (experiment 1) and September 29, 2015 (experiment 2). Canes from the field were cut approximately 30 cm from the apex, placed in water, and transported to a cold room (4 °C) where they were used within 4 h of collection. Fruit were placed in bags, incubated at room temperature (22 °C) for 24 h (12:12 L:D) and then placed at 17 °C; these conditions were meant to mimic mild (near room temperature) storage temperatures, as would occur in retail, which are highly conducive to disease development. CFR development was evaluated four days post-harvest, when all fruit were ripe. Post-harvest fruit rot incidence was quantified as the percent of fruit in each replicate the developed CFR symptoms.

Presence of D. suzukii *larvae in CFR-affected berries*. To develop baseline data on potential for these two pests to interact, surveys were conducted at two farms over two years to determine whether CFR-affected berries could be infested with healthy *D. suzukii* larvae. The survey was conducted using the same design and timeline as above. After visual examination for CFR, each fruit was gently macerated and visually inspected for presence of *Drosophila* larvae (Fig. 1c). To confirm larvae species identity, raspberries from these sites were monitored for *D. suzukii* infestations from 7/2/15 to 10/8/15, and a subset of larvae (N = 85) were reared to adulthood following methods described in Hamby et al. 2012; 100% of larvae were

D. suzukii. Association with *D. suzukii* was quantified as the percent of *Cladosporium*-infected berries with *Drosophila* larvae. Correlation between *D. suzukii* and CFR incidence was analyzed using linear regression.

Presence of *Cladosporium* species in frass of Drosophila suzukii *larvae collected in the field*. To determine whether *Cladosporium* propagules are present within *D. suzukii* larvae in red raspberry fields, 2nd to 3rd instar larvae were removed from fruit (asymptomatic for *Cladosporium* or any other fruit rot) between August 8 and November 2, 2015, using sterile Featherweight forceps (BioQuip, Rancho Dominguez, CA). In total 12 and 14 larvae were collected from the Woodbine, MD and Germantown, MD sites respectively. Gut microorganisms were isolated following methods described in Lewis et al. (2018) and Hamby et al. (2012). Briefly, each larva was surface sterilized by submergence in sterile autoclaved distilled water (~10 s), followed by 70% ethanol (~10 s), and a final rinse in autoclaved water (~10 s). Each larva was then placed on a Rose Bengal +0.1 g/L Chloramphenicol (RBCA) agar plate for 30–60 min (depending on larval activity), where it would crawl and defecate frass. The frass contained live fungal propagules which grew into visible cultures and were enumerated as the number of colony forming units / larvae. All *Cladosporium*-like colonies were then transferred to 10% PDA for identification. All larvae were transferred to artificial diet and reared to adulthood to confirm identity as *D. suzukii* following the methods described in Hamby et al. 2012.

Larval frass plates were evaluated at 3–5 day intervals for 20 days. All fungal colonies resembling *Cladosporium* were identified to genus based on spore morphology as described in Barnett and Hunter (1998). Incidence of larval association was quantified as the percent of larvae with one or more *Cladosporium* isolate. Downstream analysis of species identity was conducted for a subset of isolates as described below.

Statistical analyses for surveys The percent of fruit per row with *Cladosporium* fruit rot symptoms pre-harvest were analyzed with multi-way ANOVA and Tukey's means comparison (using R ×64 3.3.2 and Rcmdr plug in). Location in row, site and date were treated as fixed effects; experiment and row were treated as random effects. Analyses were also conducted for each site separately. The percent of fruit in each replicate that developed CFR symptoms post-harvest were analyzed with a one-way ANOVA. Fruit maturity was treated as a fixed effect and both experiment and block were random effects. Incidence data were arcsine-squareroot transformed prior to analyses. Data for replicate experiments were combined in the absence of significant experiment x fixed effect interaction.

Characterizing *Cladosporium* species associated with raspberry fruit rot and frass from field-collected *Drosophila suzukii* larvae

Curating *Cladosporium* isolates from CFR-affected fruit. Between August and September 2015, putative CFR-affect fruit were collected from three farms (Woodbine MD, Germantown MD, and Salisbury MD) (4–10 fruit / site). The fruit were stored at 4 °C for up to five days prior to isolation, and then incubated for two to four days in plastic bags on moist towels (12:12 L:D, 24 °C). Mycelium and spores emerging from the berry were aseptically transferred directly to 10% Potato Dextrose Agar +0.1% Tetracycline (PDA + Tet). All *Cladosporium* isolates were pure cultured using a single hyphal tip and identified to genus based on morphological characteristics as described in Barnett and Hunter (1998). Species identity was analyzed as described below for fifteen *Cladosporium* isolates from fruit, with 4–7 isolates from every site (each isolate representing a different fruit sample) (Table 2).

Curating *Cladosporium* isolates from field-collected Drosophila suzukii *larvae*. *Cladosporium* isolates recovered from the frass of *D. suzukii* larvae in the above study were transferred to PDA + Tet in an effort to prevent contamination of the colony by bacteria, yeasts and other hyphal fungi. At this stage a majority of isolates were lost since they were contaminated by the abundant yeasts present in the frass. All remaining isolates were pure cultured using a single hyphal tip of a single spore. In total, we were able to establish four pure cultures from the Germantown site and one from the Woodbine site; each culture represented a distinct larva. Species identity of all five isolates were analyzed as described below.

Species identification: BLAST-analysis DNA was extracted from 5 to 7 day old mycelium using Prepman Ultra (Applied Biosystems), and both Actin (ACT) (ACT 512F / ACT 783R, 207–230 bp region) and Translation Elongation Factor 1-α (EF) regions (EF728F / EF2R, 370–600 bp region) were amplified as described in Bensch et al. 2010. Amplifications were performed using GoTaq green master mix (Promega) on a Bio-Rad

C1000 Touch thermalcycler. ACT amplification consisted of: 94 °C for 5 min; 45 cycles of 94 °C for 45 s, 52 °C for 30 s, 72 °C for 90 s; 72 °C for 6 min. EF amplification consisted of: 95 °C for 8 min; 40 cycles of 95 °C for 15 s, 55 °C for 20 s, 72 °C for 1 min; 72 °C for 5 min.

After successful amplification was confirmed by agarose gel electrophoresis, forward and reverse amplicons were purified using the MO Bio UltraClean PCR Clean-Up Kit and submitted to Genewiz for sequencing. Both forward and reverse sequences from each isolate were combined using CLC Workbench 7 software to create trimmed contigs. Sequences were then compared using BLAST searches against accessions from ex-type cultures and other voucher specimens in the GenBank nucleotide collection to determine isolate identity. Most BLAST searches employed the default "megablast" search algorithm, but for some EF sequences, switching to either "blastn" or "discontinuous megablast" was necessary in order to ensure that searches were continuous over gap-rich intronic regions.

Phylogenetic analysis Sequence data from a subset of the *Cladosporium cladosporioides* species complex containing the relevant species was assembled using the list of strains included with the GOPHY 1 *Cladosporium* section (Marin-Felix et al. 2017). Sequences of relevant voucher specimens and other strains that were close matches during the BLAST searches (Table S2) were added for a total of 60 taxa, each represented by both loci (although several taxa were missing a section of the 5' EF locus); sequences obtained from 20 isolates in this study (15 from fruit, 5 from frass) were added for a total of 80 taxa.

The EF and ACT loci were first roughly trimmed and then aligned separately using MAFFT-E-INS-I (Katoh and Standley 2013). Alignments were not manually adjusted or filtered, although the ends of some sequences that terminated within the alignment were trimmed, as were the alignment borders. FastGap (Borchsenius 2009) was used to extract informative gaps from each locus and these were encoded as separate subsets and concatenated to each alignment. The two alignments were combined using FasConCAT (Kück and Meusemann 2010), and submitted to PartitionFinder2 (Lanfear et al. 2016), partitioning introns, exons and gaps separately (S3 Table). Exons were not further subdivided by codon position because this would have resulted in several subsets with a lack of informative characters. The partitioning and model scheme chosen by PartitionFinder2 (S3 Table) was used for phylogenetic inference based on maximum likelihood (Garli 2.01, Zwickl 2006) and Bayesian likelihood (MrBayes 3.2.6, Ronquist et al. 2012) approaches; trees were inferred and illustrated using methods of Bourret et al. (2018) and the two loci were also analyzed individually using the same procedures (S1 and S2 Figures).

Characterizing *Cladosporium* species as fruit rot pathogens of red raspberry

Characterizing pathogenicity of *Cladosporium* species from raspberry fruit rot. Pathogenicity was evaluated for three *C. pseudocladosporioides* isolates (SL 895, SL 905, SL 1027), one *C. cladosporioides* isolate (SL 902), and two *C. anthropophilum* isolates (SL 906 and SL 1025) on detached ripe red raspberries (Table 2). Because the latter two species were closely related and could not be unambiguously separated in the phylogenetic analysis, isolates of *C. anthropophilum* and *C. cladosporioides* were grouped together for the purposes of analyzing pathogenicity trials. Two inoculation treatments were implemented on individual fruit (berries are a composite of multiple fruits, referred to as drupes): (1) intact drupe inoculation (no wound inoculation) and (2) inoculation of a wounded drupe (wound inoculation). Non-inoculated intact drupe and non-inoculated wounded drupe treatments served as negative controls, for a total of 14 treatments. Three berries (subreplicates) were allocated to each treatment in each of the three replicate incubators, for a total of nine berries per treatment, and this experiment was conducted twice.

In preparation for inoculations, red raspberries were surface disinfested by soaking in 0.1% Tween 20 for 2 min, 70% ETOH for 30 s, and 0.1% NaClO for 2 min, and then the surface was air dried at room temperature in a flow hood until there was no residual moisture (~20 min). Inoculations were conducted using a spore suspension wherein five to seven day-old cultures (grown on PDA under ambient light at 27 °C) were flooded with sterile 0.5% KCl + 0.1% Tween 20 solution, spores were dislodged with a sterilized glass rod and poured through sterile cheese cloth into a 50 ml falcon tube. Spore concentration was quantified using a hemocytometer and adjusted to 10^7 spores / ml. Inoculum was stored at 4 °C and used within 24 h. To determine viable inoculum loads at the time of inoculation, aqueous dilutions of four subsamples / isolate were spread over the surface of 10% PDA plates (Aegerter

and Gordon 2006) at a target dose of 25 spores per plate. Across both experiments, mean viable spore concentration for *C. pseudocladosporioides* isolates SL 895, SL 905, and SL 1027 respectively was $8.0 \times 10^6 \pm 3.8 \times 10^6$, $7.0 \times 10^6 \pm 3 \times 10^6$, and $3.0 \times 10^6 \pm 2.2 \times 10^6$ spores / ml. For *C. cladosporioides* + *C. anthropophilum* isolates SL 902, SL 906, and SL 1025, viable spore concentration was $6.0 \times 10^6 \pm 5.0 \times 10^6$, $6.5 \times 10^6 \pm 4.4 \times 10^6$, and $1.0 \times 10^7 \pm 4 \times 10^6$ spores / ml, respectively. This high concentration was deemed necessary given the ephemeral nature of raspberry fruit; at lower levels, we found that disease could develop but over a slower period of time, allowing saprophytic contaminants to obfuscate results (data not shown).

Berries were placed on their sides in plastic incubators on elevated plastic sheets (sterilized with 0.5% NaClO). Approximately 1 cm of sterile water was added to the bottom of each chamber. Intact (non-wounded) berries were then inoculated by placing a 10 µl droplet of inoculum on the most apical drupe (Fig. 2). To wound inoculate, a sterile hypodermic needle was first used to create a 1 mm wound which penetrated just through the epidermis, and the inoculum droplet was placed on top of the wound. A droplet of sterile 0.5% KCl + 0.1% Tween 20 was used for non-inoculated controls (both non-wounded and wounded drupes). Incubators were sealed with Vaseline to achieve approximately 97% RH and incubators were maintained at 22–24 °C (10:14 L:D).

Disease development was evaluated as presence or absence of disease symptoms on the inoculated drupe four and seven days post inoculation. Incidence was calculated as the percent of berries on which the inoculated drupe developed symptoms, in each of three incubators. Incidence data was arcsine square root transformed to evaluate treatment differences based on ANOVA and Tukey's means comparison using R ×64 2.15.2 and the Rcmdr plug-in; experiments were combined in the absence of a significant experiment x treatment interaction ($P > 0.05$). In analyses, isolate and inoculation method were treated as fixed effects and block (incubator) and experiment were treated as random effects. As noted above, because *C. anthropophilum* and *C. cladosporioides* were closely related and could not be unambiguously separated, isolates were grouped together for analysis.

Pathogenicity of *Cladosporium* cladosporioides and *Cladosporium* pseudocladosporioides *isolates from larval frass.* To determine whether *Cladosporium* species from larval frass could initiate fruit rot, we evaluated pathogenicity of one isolate each of *Cladosporium cladosporioides* (SL 1024) and *Cladosporium pseudocladosporioides* (SL 1020) from frass of *D. suzukii* larvae, with known pathogenic isolates from berries as positive controls (*C. cladosporioides*: SL 902; *C. pseudocladosporioides*: SL 905). Non-inoculated fruit (sterile 0.5% KCl + 0.1% Tween 20) were included to control for background contaminants. As above, two inoculation treatments were implemented: (1) intact drupe inoculation (no wounding) and (2) inoculation on a 1 mm wound. Three berries (sub-replicates) were allocated to each of the ten treatments in each of three replicate incubators, for a total of nine berries per isolate per treatment. The experiment was conducted twice.

The inoculation methods were the same as described above. Based on spore viability assessment, mean inoculum densities were $9.8 \times 10^6 \pm 6.3 \times 10^5$, $8.2 \times 10^6 \pm 7.4 \times 10^5$, $1.9 \times 10^7 \pm 9.9 \times 10^5$, $1.0 \times 10^7 \pm 8.3 \times 10^5$ viable spores / ml in the SL 902, SL 905, SL 1020, and SL 1024 isolate treatments respectively, across both experiments. Disease development was evaluated as the presence or absence of disease symptoms on the inoculated drupe at three and six days post inoculation. Incidence

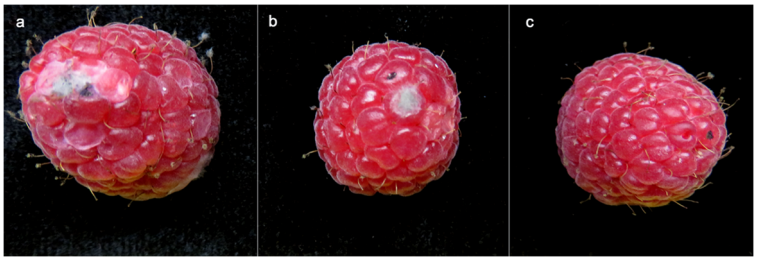


Fig. 2 Fruit rot development six days after inoculation of *C. pseudocladosporioides* isolates (**a**) from *Drosophila suzukii* larval frass and (**b**) raspberry fruit, or (**c**) treated with sterile 0.5% KCL + 0.1% Tween 20 (negative control). Similar results were obtained from *C. cladosporioides* inoculations

was calculated as the percent of berries on which the inoculated drupe developed symptoms, in each of three incubators. Analyses were conducted as previously described.

Results

Field surveys: *Cladosporium* fruit rot surveys in fall red raspberry fields and presence of *Drosophila suzukii* in CFR-affected fruit

Pre-harvest CFR incidence Cladosporium fruit rot (CFR) was present at all sites surveyed (Table 1). Minor fruit rot was often observed as green mycelium on one to three drupes (Fig. 1a), which could expand to cover a greater portion of the berry surface and induce tissue necrosis (Fig. 1b). In some cases, there were no apparent external symptoms (Fig. 1d) but when removed from the stem, fungal hyphae was prolifically colonizing the inner core of the fruit cluster (Fig. 1e). In 2015 field surveys, 0.8 ± 0.8% and 2.5 ± 1.3% of fruit had signs and symptoms of CFR at Germantown and Woodbine sites respectively, and in 2016 (Germantown site only) 32 ± 4.2% of fruit were affected by CFR (Table 1). Based on multi-way ANOVA, there was no significant Date x Site x Canopy Location interaction ($P = 0.08$), so data were combined for analyses. There was a significant effect of date ($P < 0.001$) but not of site ($P = 0.65$) or location within the canopy ($P = 0.15$) based on ANOVA. However, when analyzed separately for each site and date, 2016 surveys at the Germantown site revealed a significant effect of canopy location on fruit rot incidence ($P = 0.02$), reflecting significantly lower CFR incidence in fruit located in the upper exterior of the canopy compared to fruit in the lower exterior and interior canopy center (Table 1).

Table 1 Incidence and canopy distribution of *Cladosporium* fruit rot and *D. suzukii* in Maryland red raspberry fields across two years[a]

Location[b]	Date	Canopy location[c]	N (row)[d]	CFR incidence[e]	D. suzukii incidence[f]	D. suzukii -CFR co-occurrence[g]
Germantown	8/28/2015	Lower, exterior	3	0% a	3.3% ± 3.3%	0%
(var. Carol)		Top, exterior	3	3.3 ± 3.3% a	6.7% ± 6.7%	0%
		Center, exterior	3	0% a	6.7% ± 3.3%	0%
		Center, interior	3	0% a	26.7% ± 8.8%	0%
Site Total			12	0.83 ± 0.83%	10.8 ± 1.3%	0%
Germantown	9/28/2016	Lower, exterior	3	33.3 ± 8.8% b	20 ± 10%	6.7 ± 3.3%
(var. Himbo Top)		Top, exterior	3	13.3 ± 3.3% a	10 ± 5.8%	3.3 ± 3.3%
		Center, exterior	3	43.4 ± 6.7% ab	6.7 ± 3.3%	3.3 ± 3.3%
		Center, interior	3	36.7 ± 3.3% b	46.7 ± 12%	13.3 ± 6.7%
Site Total			12	31.6 ± 4.2%	20.8 ± 2%	6.7 ± 0.8%
Woodbine	8/28/2015	Lower, exterior	3	6.7 ± 3.3% a	33.3 ± 8.8%	3.3 ± 3.3%
(var. Jaclyn)		Top, exterior	3	0% a	3.3 ± 3.3%	0%
		Center, exterior	3	0% a	26.7 ± 8.8%	0%
		Center, interior	3	3.3 ± 3.3% a	13.3 ± 8.8%	0%
Site Total			12	2.5 ± 1.3	19.2 ± 1.4%	0.8 ± 0.8%

[a] A small subset of the data presented here is also presented in the Lewis et al. 2018 concurrent study of whole fungal communities associated with *D. suzukii* frass

[b] Var: variety

[c] Canopy locations. Lower: lower fruit in the canopy exterior (poor fungicide coverage and low to moderate humidity); Top, exterior: topmost fruit of the canopy exterior (optimal fungicide coverage, low humidity); Center, exterior: topmost fruit of the hedge center (approximately 30–60 cm from the edge; moderate to optimal fungicide coverage, low humidity); and Center, interior: lower fruit of the hedge interior (poor fungicide coverage and high humidity)

[d] Three rows/site, 10 fruit/canopy location in each row, for a total of 40 fruit/row

[e] *Cladosporium* fruit rot (CFR) incidence based on the mean ± SE percent of fruit / location or total, which exhbited symptoms of (Fig. 1). Means with the same letter are not significantly different based on Tukey's means comparison, when data for each site and date analyzed seperately

[f] *Drosphila suzkii* incidence, based on mean ± SE percent of fruit / location or total, which contained *D. suzukii*-like larvae

[g] *Cladosporium* fruit rot-*D. suzukii* co-occurance, based on the mean ± SE percent of all fruit ($n = 10$) with both *Cladosporium* fruit rot (CFR) and *D. suzukii* (SWD) larvae

Post-harvest CFR incidence Four days after harvest (2015, Woodbine site), CFR developed in 51% ± 11% of fruit harvested when red-ripe and 16% ± 9% of fruit harvested when under-ripe (pink) (Fig. 3). Maturity at harvest significantly affected CFR incidence (P = 0.02), reflecting a 68% decrease in fruit rot incidence when harvested under-ripe, compared to when harvested red-ripe [experiments were combined in the absence of a significant experiment x maturity at harvest interaction ($P = 0.66$)] (Fig. 3).

Presence of D. suzukii *larvae in CFR-affected berries.*. *Drosophila suzukii* larvae were observed within CFR-affected berries (Fig. 1c). At the Woodbine field (2015) and the Germantown field (2016), fruit rot and larvae co-occurred in 0.8 ± 0.8% and 6.7 ± 0.8% of total examined berries, respectively (Table 1, Fig. 1c). However, when looking only at those berries with CFR signs and symptoms, 33% contained *D. suzukii* larvae at the Woodbine site (2015) ($n = 3$) [see also Lewis et al. 2018] and 21% of berries ($n = 37$) at the Germantown site (2016). In most cases, larvae were clearly alive and developing in the *Cladosporium*-infested fruit. Co-association was not observed in the Germantown field in 2015, which may be related to lower overall occurrence of both *Cladosporium* fruit rot and *D. suzukii*, compared to the other site and date (Table 1). In regression analysis of incidence data across all sites, there was a weak positive correlation between *D. suzukii* and CFR incidence ($R^2 = 0.21$; $y = 0.2104x + 0.1449$).

Presence of *Cladosporium species in frass* of Drosophila suzukii *larvae collected in the field. Cladosporium* isolates were recovered from frass of 71% of larvae collected from the Germantown site ($n = 14$), with an average of 4.6 colony forming units (CFUs) per infested larva (see also Lewis et al. 2018). *Cladosporium* was recovered from frass of 25% of larvae from the Woodbine site ($n = 12$), with an average of 2.33 CFUs / infested larva (see also Lewis et al. 2018). 100% of the larvae carrying *Cladosporium* propagules were successfully reared to adulthood and were confirmed as *D. suzukii*.

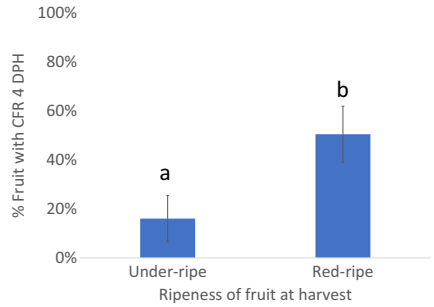


Fig. 3 *Cladosporium* fruit rot incidence four days after harvest of raspberry canes with under-ripe (pink) and red-ripe fruit. Bars with different letters are significantly different, based on Tukey's means comparison ($P = 0.03$)

Characterizing *Cladosporium* species associated with raspberry fruit rot and frass from field-collected *Drosophila suzukii* larvae

Species identification: BLAST-analysis Based on GenBank BLAST analysis of the EF region for fifteen *Cladosporium* isolates from fruit (4–7 isolates / site, three sites), six were identified as *C. cladosporioides* and nine were identified as *C. pseudocladosporioides* (S1 Table). Both species were recovered from symptomatic fruit collected from all three farms. Based on BLAST analysis of *Cladosporium* isolates from larval frass, four were identified as *C. pseudocladosporioides* (3 from Germantown and 1 from Woodbine) and one was identified as *C. cladosporioides* (Germantown). Similar results were obtained in BLAST analyses of the actin gene region.

Species identification: Phylogenetic analysis The species determinations based on the phylogenetic results were not congruent with those based on the BLAST searches. Many of the *C. cladosporioides* voucher specimens from Bensch et al. (2010) were misleading modern BLAST-derived determinations and would instead be better considered belonging to the recently-described *C. anthropophilum* based on the phylogenetic analysis, along with five of the raspberry isolates from the current study (Fig. 4). More recently, *C. anthropophilum* was re-examined by leading taxonomists and these isolates were also considered to be *C. anthropophilum* (Bensch et al. 2018). However, sequence accessions corresponding to these new *C. anthrophophilum* vouchers still bear the species name *C. cladosporioides*. Moreover, our phylogenetic results could not unambiguously separate the two species with strong support, and the two species were not mutually monophyletic in either of the single-locus trees (Supplemental Figs. 1 and 2).

Based on these results, isolates from diseased raspberries were divided into three species: *C. cladosporioides* (one isolate), *C. anthropophilum* (five isolates), and *C. pseudocladosporioides* (nine

isolates) (Fig. 4). One insect isolate was determined to be *C. cladosporioides* and the remaining four were *C. pseudocladosporioides* (Fig. 4). The *Cladosporium cladosporioides* isolates from insect and fruit were phylogenetically distinct, but a clade containing the ex-type strain of *C. pseudocladosporioides* had two pairs of closely-related insect and raspberry isolates (Fig. 4). Three of the five *Drosophila*-derived *Cladosporium* isolates were phylogenetically distinct from any raspberry strains (Fig. 4). None of the insect strains were determined to be *C. anthropophilum* based on the phylogenetic analysis.

Characterizing pathogenicity of Cladosporium species from raspberry fruit rot All isolates were able to initiate fruit rot. Symptoms were consistent with those seen in the field and consisted of a sunken dark green to black lesion coated with spores, affecting both the inoculated drupe and several adjacent drupes. By seven days post inoculation (DPI), scraping the spores away revealed a black hardened pad of hyphal growth and decayed tissue.

Without wounding, 11–50% of fruit developed rot four days after inoculation (across all isolates) and 33–66% of fruit had symptoms seven DPI. There was no effect of isolate on disease incidence when inoculated without a wound at either four or seven DPI in both experiments, with one exception where SL 1027 had lower incidence than SL 1025, SL 895 and SL 905 at four DPI in experiment 2 (Table 2).

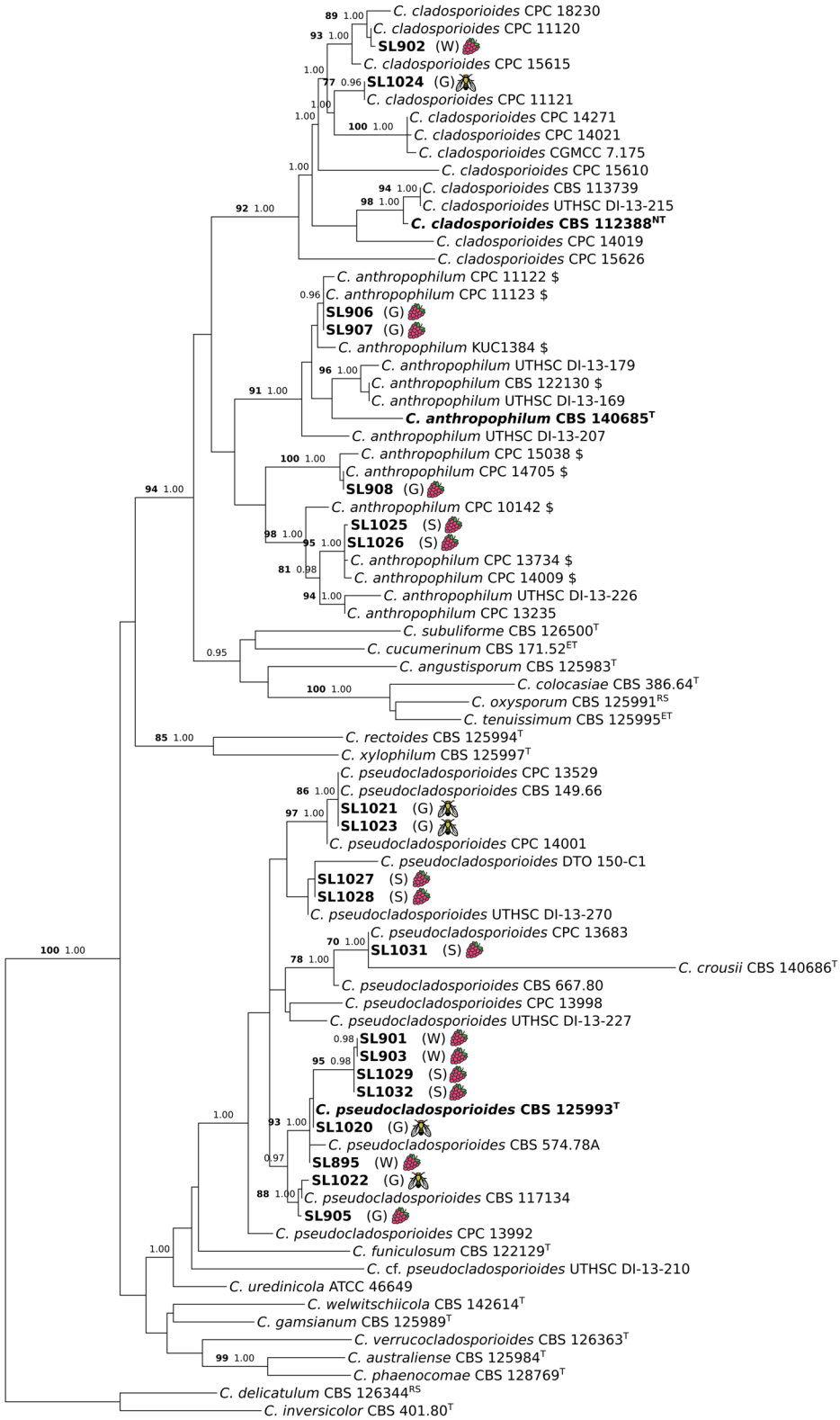
When inoculated through a wound, between 16% and 90% of fruit developed fruit rot at four DPI and 50–100% of fruit were symptomatic at seven DPI. Wounding significantly enhanced disease incidence for all isolates four days after inoculation ($P = 0.04$), with up to a 78% increase in the percent of berries with CFR compared to inoculations without a wound (Table 2). At seven DPI, disease incidence was up to 58% greater in the wounded treatment. The effect of wounding was significant for all isolates in experiment two ($P = 0.05$) but only for SL 905 in experiment one [experiments separated due to significant experiment x inoculation method interaction ($P < 0.05$); isolates separated due to significant isolate x inoculation method interaction ($P < 0.05$)] (Table 2). Isolate differences following wound inoculation were not detected at either four or seven DPI in experiment two. In experiment one, isolate differences were detected, but there were no consistent differences between four and seven DPI or between species (Table 2). Negative controls remained symptomless and were not included in the analyses.

Pathogenicity of C. cladosporioides *and* C. pseudocladosporioides *isolates from larval frass.* Both *C. cladosporioides* and *C. pseudocladosporioides* isolates from larval frass were able to cause disease on non-wound inoculated drupes (47–56% of berries infected) as well as wound inoculated drupes (70–100% of berries infected) at six days post inoculation (Fig. 2; Table 3). There was no difference in disease incidence between isolates from frass and positive controls (raspberry-derived isolates) for either *C. cladosporioides* ($P = 0.21$) or *C. pseudocladosporioides* ($P = 0.67$) (Table 3). As above, there was a significant effect of inoculation method for either *Cladosporium* species ($P < 0.05$), wherein wound inoculation increased disease incidence by 25% to 53% across isolates (Table 3). Negative controls were excluded in analyses, as no symptoms developed.

Discussion

Like many raspberry-producing regions, the Mid-Atlantic is seeing a trend towards extended production later into the fall to broaden the consumer market. The expansion of fall-bearing raspberry production increases the infection opportunities for pathogens with peak distribution in late summer and fall, such as *Cladosporium* species—a trend which is also seen in other fruit production systems, such as table grapes (Rodríguez-Rajo et al. 2005, Briceño and Latorre 2008, Swett et al. 2017). In result, *Cladosporium* fruit rot (CFR), previously only considered a minor contributor to post-harvest disease, appears to be becoming more common as both a pre- and post-harvest disease in this region.

In this study, we found two species, *Cladosporium cladosporioides* and *C. pseudocladosporioides,* as well as a putative third species, *C. anthropophilum*, causing pre-harvest fruit rot of fall bearing red raspberries at each of three Maryland farms. In field surveys, the disease was detected pre-harvest at all sites and dates,


C. cladosporioides CPC 18230
C. cladosporioides CPC 11120
SL902 (W)
C. cladosporioides CPC 15615
SL1024 (G)
C. cladosporioides CPC 11121
C. cladosporioides CPC 14271
C. cladosporioides CPC 14021
C. cladosporioides CGMCC 7.175
C. cladosporioides CPC 15610
C. cladosporioides CBS 113739
C. cladosporioides UTHSC DI-13-215
C. cladosporioides CBS 112388NT
C. cladosporioides CPC 14019
C. cladosporioides CPC 15626
C. anthropophilum CPC 11122 $
C. anthropophilum CPC 11123 $
SL906 (G)
SL907 (G)
C. anthropophilum KUC1384 $
C. anthropophilum UTHSC DI-13-179
C. anthropophilum CBS 122130 $
C. anthropophilum UTHSC DI-13-169
C. anthropophilum CBS 140685T
C. anthropophilum UTHSC DI-13-207
C. anthropophilum CPC 15038 $
C. anthropophilum CPC 14705 $
SL908 (G)
C. anthropophilum CPC 10142 $
SL1025 (S)
SL1026 (S)
C. anthropophilum CPC 13734 $
C. anthropophilum CPC 14009 $
C. anthropophilum UTHSC DI-13-226
C. anthropophilum CPC 13235
C. subuliforme CBS 126500T
C. cucumerinum CBS 171.52ET
C. angustisporum CBS 125983T
C. colocasiae CBS 386.64T
C. oxysporum CBS 125991RS
C. tenuissimum CBS 125995ET
C. rectoides CBS 125994T
C. xylophilum CBS 125997T
C. pseudocladosporioides CPC 13529
C. pseudocladosporioides CBS 149.66
SL1021 (G)
SL1023 (G)
C. pseudocladosporioides CPC 14001
C. pseudocladosporioides DTO 150-C1
SL1027 (S)
SL1028 (S)
C. pseudocladosporioides UTHSC DI-13-270
C. pseudocladosporioides CPC 13683
SL1031 (S)
C. crousii CBS 140686T
C. pseudocladosporioides CBS 667.80
C. pseudocladosporioides CPC 13998
C. pseudocladosporioides UTHSC DI-13-227
SL901 (W)
SL903 (W)
SL1029 (S)
SL1032 (S)
C. pseudocladosporioides CBS 125993T
SL1020 (G)
C. pseudocladosporioides CBS 574.78A
SL895 (W)
SL1022 (G)
C. pseudocladosporioides CBS 117134
SL905 (G)
C. pseudocladosporioides CPC 13992
C. funiculosum CBS 122129T
C. cf. pseudocladosporioides UTHSC DI-13-210
C. uredinicola ATCC 46649
C. welwitschiicola CBS 142614T
C. gamsianum CBS 125989T
C. verrucocladosporioides CBS 126363T
C. australiense CBS 125984T
C. phaenocomae CBS 128769T
C. delicatulum CBS 126344RS
C. inversicolor CBS 401.80T

◀ **Fig. 4** Most likely tree inferred from an analysis of combined elongation factor and actin, with a 80-taxon alignment of a section of the *Cladosporium cladosporioides* species complex. The tree was generated with 200 heuristic best tree searches with Garli 2.01 using a model and partitioning scheme generated by PartitionFinder2 (S2 Table). Bipartitions receiving ≥70% bootstrap support in Garli and ≥ 0.95 posterior probability in MrBayes 3.2.6 are indicated. Sequence accessions and culture collections are listed in S1 Table. Superscript letters: T, ex-type strain; ET, ex-epitype; NT, ex-neotype; RS, reference strain. $, accessions originally deposited as *C. cladosporioides*; (G), collected in Germantown, MD; (S), collected in Salisbury, MD; (W), collected in Woodbine, MD. Figure created with TreeGraph2 (Stöver and Müller 2010) and Inkscape (www.inkscape.org)

although incidence varied widely, from less than 1% to greater than 30% of berries affected. Field surveys focused on Western MD; however, the greatest yield losses were reported from a southern MD farm (Salisbury, MD). Further studies of region-wide distribution patterns and environmental effects on disease development are needed to better understand impacts of this disease in the region and identify high-risk areas and environmental conditions.

CFR incidence increased from 2.5% pre-harvest to 50% post-harvest in red ripe fruit, which is consistent with what is known of this disease in red raspberries and other fruit crops and suggests that the major economic impacts occur post-harvest (Ellis et al. 1991; Swett et al. 2017). Maturity of fruit at harvest appeared to influence disease development, perhaps due to reduced pathogen exposure of younger fruit and/or increased susceptibility of older fruit. This effect may be exploited in developing integrated methods for fruit rot management.

Despite great effort and multiple monographic works, molecular species determination in *Cladosporium* s.s. is still greatly hampered by unresolved systematic ambiguity. Generally, these disparities pose major challenges in both ascertaining etiology of diseases caused by *Cladosporium* species and exploring pathogen ecology. In the case of this study, BLAST-based determinations for many isolates as *C. cladosporioides* were not congruent with phylogenetic determinations. Bensch et al. (2010) was the first modern effort to break up the species complex based on molecular methods, but *C. cladosporioides* s.s. was still a phylogenetically wide clade with systematic ambiguities. These isolates were described as *C. cladosporioides* vouchers in the Bensch et al. (2012) monograph and were uploaded to the GeneBank database as voucher specimens. However, more recently, Sandoval-Denis et al. (2016) described several new species, including *C. anthropophilum*. By including the phylogenetically distinct UTHSC DI-13-226 as a *C. anthropophilum* voucher, that study proposed a phylogenetically wide species that appears to include many of the Bensch et al. (2010) *C. cladosporioides* voucher specimens, although few of these were included in the study. Our results suggest that many of the *C. cladosporioides* vouchers should be considered *C. anthropophilum*, which is in

Table 2 Pathogenicity of *Cladosporium* isolates from diseased raspberries based on fruit rot incidence at four and seven days after inoculation

		Disease Incidence[a]			
		4 days post inoculation		7 days post inoculation	
Isolate	Location	Non-Wound	Wound	Non-Wound	Wound
C. anthropophilum					
SL 906	Germantown, MD	17 ± 7% a, ab	33 ± 17% a, a	50 ± 18% a	50 ± 22% a, a
SL 1025	Salisbury, MD	50 ± 22% a, b	90 ± 5% b, a	66 ± 17% a	100 ± 0% b, a
C. cladosporioides					
SL 902	Woodbine, MD	17 ± 17% a, ab	16 ± 11% a, a	33 ± 17% a	77 ± 16% ab, a
C. pesudocladosporioides					
SL 895	Woodbine, MD	27 ± 16% a, b	45 ± 16% ab, a	55 ± 20% a	78 ± 11% b, a
SL 905	Germantown, MD	27 ± 13% a, b	45 ± 20% a, a	55 ± 20% a	100 ± 0% b, a
SL 1027	Salisbury, MD	11 ± 11% a, a	45 ± 20% ab, a	55 ± 16% a	100 ± 0% b, a

[a] Disease incidence (mean ± SE) quantified as the percent of berries which developed symptoms on the inoculated drupe, for two experiments combined ($n = 6$). Means ± SE separated by different letters in the same column are significantly different, based on Tukey's multiple means comparison ($P \leq 0.05$). In some analyses, experiments had to be separated due to significant isolate interaction; experimental 1 and 2 analyses are separated by a comma

Table 3 Fruit rot incidence following inoculation with *Cladosporium* isolates from *D. suzukii* larval frass six days post inoculation

Isolate	Source[b]	Disease incidence	
		Non-Wound[a]	Wound[a]
C. cladosporioides[c]			
SL 902	Raspberry	52 ± 13%	70 ± 11%
SL 1024	Frass	56 ± 10%	94 ± 6%
C. pseudocladosporioides[c]			
SL 905	Raspberry	50 ± 17%	86 ± 9%
SL 1020	Frass	47 ± 16%	100 ± 0%

[a] Disease incidence (mean ± SE) quantified as the percent of berries which developed symptoms on the inoculated drupe six days after inoculation, for two experiments combined (n = 6). Means followed by the same letter within each column are not significantly different ($P > 0.05$)

[b] There was no effect of isolate source (frass vs. raspberry), for either *C. cladosporioides* ($P = 0.21$) or *C. pseudocladosporioides* ($P = 0.67$)

[c] There was a significant effect of wounding for both *C. cladosporioides* ($P = 0.02$) and *C. pseudocladosporioides* ($P < 0.001$)

agreement with the recent work of Bensch et al. (2018). In contrast to Bensch et al. (2018), the results of our phylogenetic analysis suggest that *C. anthropophilum* is polyphyletic as currently described and more systematic work is needed. Bensch et al. (2018) also observed that *C. crousii* could not be separated from *C. pseudocladosporioides* using a multi-locus phylogeny.

Since *Cladosporium* species are not typically considered economically significant to raspberry production and have not previously been described as pre-harvest raspberry pathogens (Ellis et al. 1991), there is little management information available to control CFR in the field. Chemical treatments appear to have some, albeit limited, usefulness in controlling *Cladosporium* fruit rots (Ellis et al. 1991; Park et al. 2005; Swett et al. 2017). Field surveys indicate that the raspberry canopy many not be uniformly affected by CFR, but rather can suffer greater fruit rot impacts in certain canopy locations; further studies to identify heavily impacted canopy regions can help improve efficacy of chemical control. Researchers in the region are currently exploring canopy thinning techniques which reduce humidity to levels below those preferred for *D. suzukii* (Diepenbrock and Burrack 2016; Rice et al. 2017; Tochen et al. 2015) and also improve pesticide penetration (K. Hamby, Pers. Comm); similar methods may also be utilized to improve fruit rot management.

Cladosporium species are typically thought to be epiphytes on fruit, requiring either micro wounds (such as epidermal cracks), macro wounds, or natural openings to establish infection (Briceno and Latorre 2008). Although both *Cladosporium* species could infect drupes without wounding in our studies, epidermal wounding consistently enhanced infection success, as has been seen for CFR pathogens in other crops (Swett et al. 2017). Frugivorous insects are a major driver of fruit damage in the Mid-Atlantic (Joshie et al. 2017) and thus may influence CFR development. Spotted wing drosophila (*D. suzukii*) is one of the most prevalent and damaging insects in Maryland red raspberry fields (Hamby et al. 2014) and wounds caused by oviposition may create infection courts for *Cladosporium* to enter fruit. In addition, *D. suzukii* populations are greatest in the fall (Joshi et al. 2017), which is also when *Cladosporium* propagule production tends to be greatest (based on studies in other systems, eg. Briceno and Latorre 2008). Of note, other insects, such as stinkbugs and tarnished plant bugs can also cause fruit wounds in ripening and ripe raspberries and may play a role in disease development.

In support of potential association between *D. suzukii* and CFR, *Cladosporium* propagules were recovered from frass of 25–71% of larvae ($N = 12$–14) collected from raspberry fields. Phylogenetic analyses and subsequent pathogenicity assays revealed that fungal isolates from larval frass can cause fruit rot. Since larvae were disinfested, recovered *Cladosporium* isolates putatively represented ingested propagules that were able to successfully survive digestion and be released into the environment in frass. In a concurrent study, propagules of *Botrytis cinerea* (a common fruit rot pathogen) were either not recovered or occurred in frass at a much lower frequency (Lewis et al. 2018). This may indicate that *Cladosporium* spp., like yeast fungi (Coluccio et al. 2008; Hoang et al. 2015) are adapted to survive digestion. Although this is (to the author's knowledge) one of the first studies to suggest an association between a phytopathogenic *Cladosporium* species and an insect, there have been several studies documenting colonization of *Cladosporium* species in wide range of arthropods (Grief and Currah 2007; Trovao et al. 2013; Vega et al. 2008). Taken together, this evidence points to the potential

for co-association between pathogenic *Cladosporium* species and *D. suzukii*, warranting further studies to elucidate the nature and epidemiological significance of tri-trophic interactions between *Cladosporium* species, *D. suzukii* and raspberry fruit.

Together, these studies provide the first report of species in the *Cladosporium cladosporioides* species complex as drivers of fruit rot development and yield losses in Mid-Atlantic fall red raspberries. This study also provides observational support for an association between *Cladosporium* fruit rot pathogens and the frugivorous insect *D. suzukii*. Further studies are needed to address management concerns for *Cladosporium* fruit rot in the Mid-Atlantic, to promote continued expansion of the fall bearing raspberry industry; these efforts may be furthered, at least in part, by a better understanding of *D. suzukii* interactions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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