How Does *Botrytis cinerea* Infect Red Raspberry?

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ABSTRACT

Botrytis cinerea, causal agent of gray mold, is one of the most important pathogens affecting raspberry in the U.S. Pacific Northwest and worldwide. Fungicides are currently applied to control the disease starting from 5 to 10% bloom and continuing on a calendar basis throughout the season rather than according to inoculum level or infection risk primarily because the disease cycle on red raspberry is poorly understood. *Botrytis cinerea* was isolated from raspberry flowers and fruit sampled at seven developmental stages during each of 2015 and 2016 in a northwestern Washington raspberry field untreated with fungicides. Incidence of colonization of flowers was low (15% of total sampled flowers), but increased as fruit developed, and peaked in mature fruit (67% of total sampled fruit). In the early stages of flower development, *B. cinerea* recovery was greatest from the carpel (80% of carpels colonized) compared

with other floral organs. As fruit matured, additional floral parts were colonized by *B. cinerea*, possibly facilitating secondary internal or external infections of mature fruit. Average weekly minimum air temperature, average weekly night air temperature, cumulative rain, average weekly leaf wetness percentage, and duration of leaf wetness >90% were significantly positively correlated with *B. cinerea* colonization of raspberry in NW Washington during two seasons of this study. Our data does not support the hypothesis that the bloom period is the critical window for *B. cinerea* colonization of red raspberry and suggest that later colonization of developing fruit may be more important for gray mold development on raspberry. The outcomes of this research provide useful information for improvement of gray mold disease management strategies for red raspberry in NW Washington and elsewhere.

The ascomycete fungus, *Botrytis cinerea* Pers.:Fr., is a plant pathogen of worldwide importance, causing serious diseases of more than 200 crop species (Jarvis 1962). The most common diseases caused by this fungus are pre- and postharvest rots known as “gray mold” affecting different plant organs, including leaves, flowers, fruits, and shoots. Vegetables and small fruit crops such as raspberry, grape, strawberry, and blueberry are among the most severely affected by this pathogen. Northwestern (NW) Washington is the largest producer of processed red raspberry in the United States (U.S. Department of Agriculture, National Agricultural Statistics Service 2017), and gray mold is a major threat to the red raspberry industry in this area. Gray mold may cause up to 70% losses of harvest in a field or during postharvest storage (Dean et al. 2012; Jarvis 1962).

Chemical control remains the primary strategy to control gray mold affecting small fruit. Despite intensive calendar-based fungicide applications (five to seven sprays per growing season), raspberry growers in NW Washington routinely experience 20 to 25% annual yield losses in high disease pressure years (H. Bierlink, *personal communication*). This is due in part to our limited understanding of the infection process of raspberry plants by the pathogen including the timing of infection in relation to fruit development. Gray mold results from the colonization of mature raspberry fruit by *B. cinerea* but the initial source of the infection has long been thought to be latent or quiescent infections of flowers (Dashwood and Fox 1988; Jarvis 1962; McNicol et al. 1985). Infection of raspberry may not be restricted to the flowering stage, with the possibility of external infections of fruit occurring later in the season, possibly following wounding of the fruit (McNicol et al. 1990; Williamson and Duncan 1989) and overall greater susceptibility of mature fruit to the pathogen compared with immature

fruit (Jarvis 1962). It remains an unresolved question if floral infection is the main route of infection of raspberry fruit leading to gray mold later in the season.

Infection courts of *B. cinerea* are poorly understood. An infection study of grape flowers employing autoradiography suggested that infection occurs at the base of stamens in the receptacle area (Viret et al. 2004). Artificial inoculation of the stigmatic surfaces of grape did not lead to host defense reactions, latent infections, or disease, and latent infections by *B. cinerea* were located primarily in the receptacle (Keller et al. 2003). A study of the colonization and infection of grape floral organs by *B. cinerea* using scanning and transmission electron microscopy, detected development of most germ tubes of *B. cinerea* in the receptacle and very few in the style following inoculation of the stigma, with no evidence for *B. cinerea* germ tube growth toward the ovaries. Hyphae were also more abundant in the receptacle, regardless of the site of inoculation (Viret et al. 2004). Infection studies of strawberry revealed that *B. cinerea* colonization of the receptacle occurs through stamens (Bristow et al. 1986; Bulger et al. 1987; Dashwood and Fox 1988; Jersch et al. 1989) and that the infections may lead to the development of gray mold. Boff et al. (2003) also found petals to be an effective source of inoculum for strawberry fruit infection by *B. cinerea*. Jarvis (1962) demonstrated that detached petals were one of the most important inoculum sources for strawberry fruit infection, while infected floral parts that remain attached to the receptacle were more important in infection of raspberry. The significance of floral infections in strawberry gray mold were previously reported by Powelson (1960). However, Williamson et al. (1987) hypothesized that the significance of raspberry floral parts might be overestimated because they rarely adhere to picked fruit. McNicol et al. (1985) and Williamson et al. (1987) related *B. cinerea* infection of raspberry flowers to the pollination process that involved colonization and infection of transmitting tissues of styles and young pistils following the same route to the ovules used by pollen tubes. Examination of raspberry fruit that was previously wounded and infected by *B. cinerea* using low temperature scanning electron microscopy showed that conidia germinated only in juice surrounding wounds (Williamson and Duncan 1989).

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The shelf-life of wounded and infected fruit was also significantly reduced compared with the shelf-life of fruit that was wounded without inoculation or inoculated without wounding, suggesting that wounded ripe fruit during harvest may be another important infection court for *B. cinerea* on red raspberry later in the season. A limitation of the majority of *B. cinerea* infection studies of raspberry to date is that they employed artificial inoculation of stigmatic surfaces (McNicol et al. 1985; Williamson et al. 1987) which may potentially bias our understanding of the infection process. Artificial inoculation of raspberry flower stigmas with *B. cinerea* conidia (Williamson et al. (1987)) under different environmental conditions resulted in infection of carpels in the greenhouse and laboratory, but not in the field. These authors hypothesized that a stigmatic infection route may be more important in warmer raspberry growing regions compared with NE Scotland. In a field study employing natural inoculum, Dashwood and Fox (1988) plated sterilized samples of different developmental stages of raspberry flowers and fruit and rarely isolated the fungus from recently-opened flowers or receptacles before harvest. However, the fungus was commonly isolated from green and ripe fruit. This study also reported high levels of colonization of stamens and styles at all developmental stages. Carisse et al. (2014) suggested that disease cycles of *B. cinerea* on raspberry, strawberry and grape may be similar despite the fact that duration and timing of flowering varies significantly among these plants. Strawberry and grape have limited bloom periods relative to raspberry, which blooms over a much longer period with flowers still being formed at harvest (Ellis et al. 2008). Therefore, *B. cinerea* infection courts on raspberry may differ significantly compared with other small fruit crops and may be strongly affected by host phenology and the local environment in which raspberry plants are grown. More research is needed to determine which raspberry flower organs are infected at different flower and fruit developmental stages throughout the growing season.

The presence of free water is crucial for floral infection of raspberry and strawberry by *B. cinerea* (Bulger et al. 1987; Jarvis 1962; Williamson and Duncan 1989). Xu et al. (2000) found that strawberry flower infection was favored by low daytime vapor pressure deficits (high moisture level) and night temperatures up to 20°C. Inoculation studies of *B. cinerea* involving several hosts (Bulger et al. 1987; Ciliberti et al. 2015; Cole et al. 1996; Williamson et al. 1995) have shown a similar dependence on the presence of free water and high relative humidity in the infection process. The optimum temperature for infection of strawberry flowers was 20°C, with 100% of flowers infected after 24 h of leaf wetness (Bulger et al. 1987). Frequency of flower and fruit infection decreased dramatically above 25 and below 15°C for all wetness durations, indicating that disease development was highly dependent on both temperature and leaf wetness duration (Bulger et al. 1987).

Fig. 1. Developmental stages of raspberry flowers and fruit: S1, closed green buds; S2, half-open flowers; S3, open flowers; S4, half-developed fruit; S5, green fruit; S6, immature fruit; and S7, mature fruit.

Similarly, longer periods of leaf wetness were required for infection of grape when temperatures moved away from the optimum for *B. cinerea* (Nair and Allen 1993). Ciliberti et al. (2015) reported that wounding of grape berries prior to inoculation with *B. cinerea* mycelium increased gray mold incidence 1.5 to 5 times relative to incidence on unwounded grape berries under suboptimal conditions. Under optimum (continuous 100% relative humidity or 36 h wetness duration) or unfavorable (65% relative humidity or 3 h of wetness duration) conditions, wounding did not have the same effect. The authors speculated that under intermediate humidity or wetness conditions, wounds may facilitate infection. The potential role of wounding in the infection process and gray mold development may be especially important for mechanically harvested fruit which characterizes processed raspberry production in the U.S. Pacific Northwest. Jarvis (1964) reported a high correlation of raspberry gray mold incidence with preharvest durations of relative humidity >80% and preharvest rainfall. The abovementioned strawberry gray mold model (Xu et al. 2000) did not consistently predict the incidence of flower infections of raspberry grown in tunnels (Xu et al. 2012), suggesting that local environment (differences in temperature and humidity between open fields versus tunnels) plays important role in the infection by *B. cinerea*. Model predictions also over-estimated the incidence of raspberry flower infection. The authors hypothesized that additional factors, such as inoculum concentration, may affect *B. cinerea* infection of raspberry plants. Carisse et al. (2018) found a significant correlation between red raspberry crop losses, average relative humidity and average airborne inoculum concentration before bloom. To our knowledge, the influence of local environmental conditions on the development of gray mold of red raspberry has never been investigated in the U.S. Pacific Northwest, and critical information about the epidemiology of this disease in this region remains limited.

The objectives of this research were to determine the infection pathways of *B. cinerea* on raspberry under natural conditions in the field. The specific objectives were to (i) investigate the dynamics of red raspberry flower and fruit infection by *B. cinerea*, (ii) study the role of flower and fruit organs in raspberry fruit infection, and (iii) relate *B. cinerea* colonization of red raspberry to environmental conditions in NW Washington.

MATERIALS AND METHODS

Sampling. Raspberry flowers and fruit were sampled from a single row of red raspberry cultivar Meeker located at the Washington State University's Northwestern Research and Extension Center, Mount Vernon, WA (GPS coordinates 48.439138N, -122.387459W) at multiple times during 2015 and 2016 to assess the timing of *B. cinerea* infection. These raspberries were not treated with fungicides or insecticides in either year. Over the course of the experiment, 4,640 flowers and fruit (720 in 2015, and 3,920 in 2016) were sampled and evaluated from 28 April to 15 July 2015 and from 22 April to 18 July 2016. Seven phenological stages of flowers and fruit (Fig. 1) were sampled at weekly intervals.

Recovery of *B. cinerea*. Forty samples of each developmental stage (S1, closed green buds; S2, half open flowers; S3, open flowers; S4, half developed fruit; S5, green fruit; S6, immature fruit; and S7, mature fruit) (Fig. 1) that was present in a field at each sampling date were obtained from the experimental plot. Twenty were selected randomly and surface-disinfested in 70% alcohol for 10 s, followed by 1 min in a 1% NaOCl solution, and washed in deionized water three times for 1 min each time (surface-disinfested [SD]) and 20 were left not disinfested (non-surface-disinfested [NSD]). SD and NSD samples were plated in Petri dishes containing 1.5% water agar (WA) or *Botrytis* selective medium (BSM) (Kritzman and Netzer 1978). Four to six days after growth of putative *B. cinerea* cultures, isolates were transferred to Petri dishes containing half-strength potato dextrose agar (PDA) medium. Genus and species identification was based on morphological characters,

including overall color of the culture, conidiophore and conidial morphology, and sclerotium production. Four putative *B. cinerea* isolates were selected and species identification verified using partial DNA sequences of the nuclear ribosomal internal transcribed spacer (ITS) region, heat-shock protein 60 gene (HSP60), and glyceraldehyde-3-phosphate dehydrogenase gene (G3PDH). DNA was extracted from frozen mycelium of each of four isolates using the DNeasy PowerSoil Kit (Qiagen, Germantown, MD) following the manufacturer's protocol. The primer pair ITS1/ITS4 was used to amplify the ITS rDNA (White et al. 1990). Primer pairs G3PDHfor/G3PDHrev and HSP60for/HSP60rev were used to amplify partial fragments of the G3PDH and HSP60 genes, respectively (Staats et al. 2005). PCR reactions were each performed in a 25 μ l volume containing 1 μ l of 10 ng of fungal genomic DNA, 2.5 μ l of 10 $\times$ Taq buffer solution, 0.5 μ l of 10 mM dNTP stock, 0.5 μ l of 50 μ M of each of the forward and reverse primers, and 0.25 μ l of Taq DNA Polymerase (GenScript USA Inc., Piscataway, NJ). PCR reactions were carried out in a Bio-Rad MyCycler thermal cycler (BioRad Laboratories, Hercules, CA) with the following conditions: initial preheating for 3 min at 94°C; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s (except for an annealing temperature for the primer pair amplifying partial *G3PDH* was 64°C for 45 s), and extension at 72°C for 1 min; and a final extension at 72°C for 7 min. PCR products were purified with ExoSAP-IT (Thermo Fisher Scientific, Waltham, MA) and sequenced by ELIM Biopharmaceuticals (Hayward, CA). Sequences were aligned and compared in Geneious v8.1.8 (Biomatters Inc., Newark, NJ). Species identification was confirmed using a BLAST search to the NCBI databases with a 99.8 to 100% match to the sequences of accessions AJ705000 (*G3PDH*); CP009811 (*HSP60*); and KR080290 (ITS rDNA).

Dynamics of *B. cinerea* colonization of red raspberry flowers and fruit. To determine the timing of flower and fruit colonization by *B. cinerea* and to assess the connection between flower colonization and fruit colonization, recovery of *B. cinerea* was recorded for developmental stages present in the field at each sampling date. Fungal recovery from SD samples was assumed to represent internal colonization while recovery from NSD samples was assumed to represent external colonization of raspberry flowers and fruit. Fungal colonization was tracked as the presence or absence of *B. cinerea* from each of 20 SD or 20 NSD flower or fruit samples for each developmental stage at each sampling event. To further investigate the relationship between flower and fruit colonization and phenology, five newly opened flowers were marked in the field at each sampling date in 2016 and their development monitored by taking photographs of each flower every two days throughout the season. This facilitated tracking the timing of fruit development of each cohort of flowers in relation to *B. cinerea* colonization based on the phenology of the raspberry plants.

To estimate potential interference by the host defense system on rates of colonization and recovery of *B. cinerea* at flower stages S1 (closed green buds) to S4 (half-developed fruit), 70 additional SD samples were collected in the 2016 sampling season for each of the stages and air dried in a laminar flow hood for 1 h, and frozen at -80°C for 1 month to kill the host tissue. Frozen samples were then plated on BSM to record *B. cinerea* colonization incidences.

Pathways of *B. cinerea* colonization of red raspberry. In order to determine potential pathways of *B. cinerea* colonization of raspberry flowers and fruit, all SD samples were dissected into the following parts: carpels, stamens, sepals, pedicle, receptacle, and petals. Each part was incubated separately on WA or BSM. Colonization of each part by *B. cinerea* was recorded as the percentage of samples from which the pathogen was recovered using the protocol described above.

Association of environmental conditions with *B. cinerea* colonization of red raspberry. To determine the effect of the local environmental conditions on the colonization and infection process of raspberry flowers and fruit, four environmental variables

were monitored throughout both field seasons. Air temperature, relative humidity and leaf wetness were monitored with a HOBO Micro Station H21-002 weather station (Onset Computer Corporation, Bourne, MA) located in the raspberry field sampled. Weekly averages of each variable were estimated with HOBOWare PRO v.3. x software. Average weekly rainfall data were obtained from the Washington State University Agweathernet station 63 (Mount Vernon, WA, 48.44°N, -122.39°W).

Data analyses. Data analyses was performed using SAS University Edition (v. 9.4TSM4, SAS Institute Inc.). Logistic regression was used to test the effect of raspberry developmental stage on colonization of plant tissues by *B. cinerea*. Since *B. cinerea* colonization of raspberry flowers and fruit differed significantly between 2015 and 2016, a separate analysis was performed for each season and each surface-disinfestation treatment at each sampling date using PROC LOGISTIC. Colonization of each sample by *B. cinerea* was treated as a binary response (0 = absence of colonization by *B. cinerea*, and 1 = presence of colonization by *B. cinerea*) with the probability modeled to “colonization = 1”.

Statistical models of *B. cinerea* colonization detected on SD and NSD samples over sampling times were chosen based on significance of Wald χ^2 for each parameter using the LOGISTIC procedure. For both surface-disinfestation treatments, developmental stage was treated as a main effect (for SD samples Wald χ^2 = 314.9, P < 0.0001; for NSD samples Wald χ^2 = 274.9, P < 0.0001). The incidence of *B. cinerea* colonization was estimated as the means of the predicted probability of colonization for each stage and sampling date using the PROC MEANS procedure. Significance testing was performed using maximum likelihood with an odds ratio and a 95% significance level. Odds ratio is a relative measure (Agresti 2002) that allowed the comparison of a group of samples colonized by *B. cinerea* to a group of samples not colonized by *B. cinerea* and was estimated as the probability of colonization divided by the probability of no colonization. At weeks 5 (26 May), 6 (1 June), 7 (8 June), and 8 (15 June) in 2015 and weeks 5 (23 May), 6 (30 May), and 7 (6 June) in 2016, some developmental stages had zero colonization by *B. cinerea*. This led to quasi-complete separation of data points (Albert and Anderson 1984) and maximum likelihood estimates could not be made. To obtain maximum likelihood estimates of fungal colonization at these sampling dates, exact logistic regression (Hirji 2005) was used.

Statistical models of *B. cinerea* colonization of morphological parts (carpel, stamen, sepal, pedicle, receptacle, and petal) at each sampling stage were chosen based on the significance of Wald's χ^2 for each parameter using the LOGISTIC procedure as described above. Statistical analysis was performed for each year separately. Stage, morphological part and their interaction were treated as main effects. In 2015, two morphological parts (pedicle and receptacle) had zero colonization at multiple stages and sampling events and this led to quasi-complete separation of data points during the logistic regression analysis. To obtain maximum likelihood estimates of fungal colonization of each morphological part at each developmental stage in 2015, these two parts were excluded from the statistical analysis for 2015. Major parts that were colonized at each stage, the increase in frequency of the colonization of multiple morphological parts with raspberry fruit development and maturation and overlap in colonization among parts were illustrated through the construction of Venn Diagrams using the package “VennDiagram” version 1.6.17 in R Studio version 1.0.136.

Correlation coefficients between variables and their associated significance levels were estimated using the CORR procedure for Pearson's correlation coefficient.

RESULTS

Dynamics of colonization of red raspberry flowers and fruit by *B. cinerea*. Sampling was carried out weekly for 12 weeks in 2015, whereas the 2016 sampling season lasted 13 weeks.

In both years, *B. cinerea* colonization of SD samples increased as the season progressed, with the first significant increase at the open flower stage (S3) relative to closed green buds (S1) or half-open flowers (S2) (Fig. 2). Colonization of SD open flowers (S3) remained limited throughout the season, and only 6 and 15% of SD open flowers were colonized by *B. cinerea* during the 5-week flowering period in 2015 and 8-week flowering period in 2016, respectively. *B. cinerea* colonization detected from SD samples increased significantly as the fruit developed averaging 15 and 40% from green fruit (S4 to S5) in 2015 and 2016, respectively. Colonization of ripe fruit (S7) was significantly greater than that of green fruit or flowers reaching an average of 27 and 67% at the mature fruit stage of the total number of sampled fruit in 2015 and 2016, respectively.

No differences in rates of recovery were observed between frozen flowers and fresh flowers (means of colonization percentages for S1 = 1.75% $\pm$ 0.17, for S2 = 4.25% $\pm$ 0.25, for S3 = 13.90% $\pm$ 0.53, and for S4 = 37.5% $\pm$ 0.35) suggesting that host defense response did not influence the incidence of recovery of *B. cinerea* from flowers and that internal colonization of raspberry flowers by *B. cinerea* was low.

Fig. 2. Predicted probabilities of colonization of seven developmental stages of red raspberry by *Botrytis cinerea* in 2015 and 2016. Different letters indicate significant differences in colonization incidences between stages (S1, closed green buds; S2, half-open flowers; S3, open flowers; S4, half-developed fruit; S5, green fruit; S6, immature fruit; and S7, mature fruit). Significance was estimated using the odds ratio with 95% confidence level, as described in the main text.

Phenology of red raspberry. Raspberry plants in the trial flowered continuously for 5 and 8 weeks in 2015 and 2016, respectively. Depending on the environment, plants started producing green fruit approximately 3 to 5 weeks after flowers opened. Due to this continuous phenological development, multiple developmental stages of flowers and fruit were present on a plant at the same time for much of the sampling duration. Longevity of each developmental stage differed between weeks. Based on tracking of individual flower development at each sampling event in 2016, the early stages (S1, S2, S3, and S4) were the shortest, lasting for 1 to 3 days each (Fig. 3). Stage S5 (green fruit) was the longest, averaging approximately 4 weeks. Fruit were in stage S6 (immature fruit) for 1 to 3 weeks, while fruit were in S7 stage (mature fruit) for 1 to 2 weeks.

Sampling for this study was destructive which prevented the tracking of fungal colonization of the same flower/fruit at each sampling date. However, the timing at which flowers sampled each week further turned into fruit in 2016 was determined by additional tracking of development of five newly opened individual flowers at each sampling event. Pearson's correlation coefficient between incidence of flowers colonized with the incidence of fruit colonized that developed from those flowers showed no significant correlation between the fungal colonization of flowers and later stages of flowers and fruit that developed from those flowers (Table 1).

Potential routes of *B. cinerea* colonization and infection of red raspberry. Among the 5 and 15% of flowers sampled at S3 stage that tested positive for colonization by *B. cinerea* in 2015 and 2016, respectively, the female part of the flower (carpel) was colonized most frequently by the pathogen (Fig. 4). Carpels had the greatest incidence of colonization at flower and fruit stages from S3 to S7 (range 3.3 to 12% at S3 and 31 to 44% at S7 in 2015 and 2016, respectively). As flowers developed into fruit, the incidence of *B. cinerea* colonization of stamens, sepals in both years, and receptacles and pedicels in 2016 increased (Fig. 4). Stamens were the second most commonly colonized organ (range 2.67 to 1% at S3 and 23 to 35% at S7 in 2015 and 2016, respectively) after carpels, and incidences of colonization of stamens were not significantly different from that of carpel colonization at stages S3, S4, S6, and S7 in 2015 and S6 and S7 in 2016. Pedicels showed the lowest incidence of colonization at all stages sampled (range from 0% at S3 to 7% at S7 in 2016) (Fig. 4). Due to low colonization of pedicels and receptacles by *B. cinerea* in 2015, these parts were not included in the statistical analyses for that year.

As raspberry fruit developed, the frequency of *B. cinerea* colonization of multiple floral parts from the same fruit sample increased. Among the 5% of S2 samples that tested positive for *B. cinerea* colonization in 2016, 50% of receptacles, 30% of sepals, 8% of stamens, and 8% of both receptacles and sepals were colonized by *B. cinerea*. None of the carpels of the S2 samples was colonized by *B. cinerea*. Among 6% of the S3 sample that tested positive for *B. cinerea* colonization in 2015, 33% of carpels, 33% of sepals, 11% of stamens, 11% of both sepals and pedicels, and 11% of both sepals and stamens were colonized by *B. cinerea* (Fig. 5A). Among 15% of the S3 sample that tested positive for *B. cinerea* colonization in 2016, 60% of carpels, 7% of both carpels and receptacles, and 5% of both carpels and stamens were colonized by *B. cinerea* (Fig. 6B). Among 16 and 17% of the S4 and S5 samples that tested positive for *B. cinerea* colonization in 2015, 47 and 60% of carpels, respectively, 20 and 10% of stamens, respectively, 20 and 4% of sepals, respectively, were colonized by *B. cinerea* (Fig. 5B and C). Of the 13 and 20% of the S4 and S5 samples, respectively, that tested positive for *B. cinerea* colonization in 2015 the stamens and the carpels were colonized most frequently by the fungus (Fig. 5B and C). Among the 40% of the S4 and S5 samples that tested positive for *B. cinerea* colonization in 2016, 70 and 40% of carpels, respectively, were colonized by *B. cinerea*, and the percentage of colonization of multiple parts of the flowers/fruit increased at these stages (Fig. 6C and D). Among the 29% of the S6 and S7 samples

that tested positive for *B. cinerea* colonization in 2015, 40% of carpels at both stages, 20 and 10% of stamens, respectively, 20 and 7% of sepals, respectively, were colonized by *B. cinerea* (Fig. 5D and E). Of the 10 and 20% of the S6 and S7 samples, respectively, that tested positive for *B. cinerea* colonization in 2015, carpels, stamens, and sepals were all colonized in the same fruit (Fig. 5D and E). Among the 53 and 67% of samples of stages S6 and S7, respectively, that tested positive for *B. cinerea* colonization in 2016, 20% of carpels were the only organ colonized by the fungus (Fig. 6E and F). Of the 8 and 9% of the S6 and S7 samples, respectively, that were tested positive for *B. cinerea* colonization in 2016 the carpels and the stamens were colonized by *B. cinerea*. In addition, 8 and 4% of the carpels, receptacles and sepals were colonized on the same fruit by *B. cinerea* at S6 and S7, respectively. In 2% of the samples, the sepals and carpels were both colonized by the fungus on fruit at both S6 and S7. Only 4% of the S7 samples were colonized on all five parts by the fungus in 2016.

Effect of the environment on *B. cinerea* colonization of red raspberry. Weather conditions in April to July 2015 were drier and hotter than 2016 (Fig. 7). In 2015, the average daily air temperature was 16°C, with a minimum of 10°C and maximum of 34°C; daily average relative humidity (RH) was 74% with a minimum of 57% and a maximum of 92%. In 2015, total rainfall during the experiment was 51 mm spread over 11 rain events (representing 14% of the days monitored, with most rain events having <0.8 mm of rain). Daily leaf wetness was 19% with a minimum of 0.4% and a maximum of 55%, daily average number of hours with continuous leaf wetness >90% was 7 h, with a minimum of 0 h and a maximum of 14 h. In 2016, daily average air temperature was less than in 2015 (15°C, minimum 10°C and maximum 20°C) and RH was greater (79%, minimum 62% and maximum 95%). Total rainfall was 2.4 times greater in 2016 compared with 2015 (a total of 132 mm spread over 30 rainy days). Daily average leaf wetness was 41% (minimum 5% and maximum 84%), daily average number of hours with continuous leaf wetness >90% was 14h with a minimum of 2 h and a maximum of 19 h.

B. cinerea colonization of the seven flower and fruit developmental stages fluctuated throughout the season in both years

(Fig. 8) but was significantly greater overall in 2016 compared with 2015 (Wald $\chi^2 = 23.44$, $P < 0.0001$). The incidence of colonization increased in the second half of the season when fruit developed and matured. In 2015, there was no colonization of raspberry flowers by *B. cinerea* during the first 4 weeks of monitoring, possibly due to the dry weather during that period (47 mm of cumulative rain from 28 April to 10 June, $r = 0.82$, $P = 0.001$).

Among all the environmental factors measured during 2 years of study, there were significant positive correlations between *B. cinerea* colonization of raspberry flowers and fruit and average weekly night temperature ($r = 0.51$, $P = 0.0090$), average weekly minimum air temperature ($r = 0.48$, $P = 0.0147$), cumulative rain ($r = 0.75$, $P < 0.0001$), average weekly number of hours of continuous leaf wetness >90% ($r = 0.63$, $P = 0.0007$), and average weekly percentage leaf wetness ($r = 0.65$, $P = 0.0004$).

DISCUSSION

The goal of this research was to study the dynamics of *B. cinerea* colonization of red raspberry throughout plant development under natural conditions. In NW Washington, colonization of red raspberry flowers by *B. cinerea* was less than 15% in each year of the study. Colonization incidence increased over the growing season with ripe fruit showing significantly greater incidence of colonization compared with green fruit which were, in turn, colonized significantly at greater incidences than flowers. Although the primary floral organ colonized at the flowering was the carpel, colonization of additional floral organs increased significantly as the season progressed. The frequency of flower and fruit organs colonized by *B. cinerea* increased throughout the season indicating that latent infections of flowers are likely not the major route of infection leading to gray mold of fruit later in the season as previously suggested (Jarvis 1962; McNicol et al. 1985; Williamson et al. 1987). Our results suggest that the colonization of stamens and sepals may facilitate external infection of fruit later in the season, especially for mechanically harvested raspberries in this region (80% of raspberries that are grown in NW Washington are harvested mechanically) because harvesters damage fruit that stays on plants

Fig. 3. Phenology of red raspberry in 2016. Each bar represents the development of flowers sampled at each sampling event. Numbers on the top indicates sampling events.

during harvesting, creating external route for *B. cinerea* to spread from infected floral parts to wounded raspberry fruit.

Latent infections of raspberry flowers are hypothesized to be the primary route of infection leading to gray mold later in the season (Dashwood and Fox 1988; Jarvis 1962; McNicol et al. 1985; Prusky 1996; Williamson et al. 1987). In this study, incidence of raspberry flower colonization by *B. cinerea* was consistently low over two seasons, with much greater incidence of fruit colonization later in the season. Similar findings on limited colonization of flowers early in the season were reported by Dashwood and Fox (1988) in their field experiment. This does not support the floral infection/latency hypothesis. Several studies have reported active suppression of fungal growth by the raspberry plant defense system during early stages of flower development leading to latent infections (Bavaresco et al. 1997; De Lorenzo et al. 2001; Langcake 1981). We did not find any evidence that the host defense system prevented *B. cinerea* recovery from colonized flowers since freezing of the flowers prior to plating did not increase the incidence of *B. cinerea* recovered. Based on phenological observations, flowers were only open for approximately 2 days but plants flowered for 5 to 8 weeks. Such a short window for individual flowers may explain the low probability of infection on a per-flower basis. Fruit stages lasted much longer with floral parts persisting, so if conditions are conducive to *B. cinerea* colonization, fruit is potentially at a greater risk of colonization than flowers because of the longer potential window for infection. In both years of the project, *B. cinerea* colonization increased significantly during the later fruit stages and reached a maximum at the ripe fruit stage. Raspberry flowers and fruit were sampled continuously through both seasons; however, the study did not find a significant correlation between incidence of *B. cinerea* colonization of flowers and incidence of colonization of the fruit that developed from those flowers. Overall, these results do not support the hypothesis that flower infection and latent infections by *B. cinerea* are the sole route of raspberry fruit infection or responsible for the majority of raspberry gray mold in NW Washington or possibly elsewhere.

Ellis et al. (2008) reported that restriction of fungicide applications to control gray mold to the raspberry bloom period resulted in greater levels of disease compared with a spray schedule where fungicides were applied throughout the season or a program where sprays were restricted to preharvest only. Results of the Ellis et al. (2008) study support an alternative hypothesis that infection by *B. cinerea* is not restricted to the flowering stage and that late-season latent infections and/or external infections of ripe fruit may be important (McNicol et al. 1990). It may also indicate a greater susceptibility of ripe fruit versus immature fruit to the fungus (Jarvis 1962; Van Kan 2005). The amount of inoculum present in a field

may also influence the amount of gray mold. Abundant sources of *B. cinerea* inoculum, including infected plant debris, desiccated primocanes, and receptacles remaining after harvest, infected leaves and other infected plant parts are present in field throughout the raspberry growing season (Walter et al. 1997). Walter et al. (1997) identified two peaks of inoculum production, one during flowering and another at harvest. Carisse et al. (2018) found significant correlations between 7-day average airborne inoculum concentrations and average relative humidity before bloom and red raspberry fruit losses at harvest. Xu et al. (2000), studying the epidemiology of gray mold of strawberry, reported that the concentration of airborne conidia in a field varied greatly with year and significantly positively correlated with air temperature and relative humidity in some years, and negatively with current duration of surface wetness but positively with wet conditions during preceding days. Such inconsistency in the relationships of concentration of airborne inoculum with environmental conditions makes the

TABLE 1. Correlations between rates of *Botrytis cinerea* colonization of different developmental stages of red raspberry in 2016

Stage	Variable ^a	Stage ^b				
		S3	S4	S5	S6	S7
S3	<i>r</i>	1	0.49	−0.33	0.42	0.43
	<i>P</i> value		0.32	0.47	0.35	0.33
	<i>N</i>		6	7	7	7
S4	<i>r</i>		1	−0.38	0.58	−0.41
	<i>P</i> value			0.46	0.23	0.41
	<i>N</i>			6	6	6
S5	<i>r</i>			1	−0.37	−0.24
	<i>P</i> value				0.41	0.60
	<i>N</i>				7	7
S6	<i>r</i>				1	0.11
	<i>P</i> value					0.81
	<i>N</i>					7

^a *r* indicates Pearson correlation coefficient; and *N* indicates sample size for each analysis.
^b Developmental stages S3, S4, S5, S6, and S7 represent open flowers, half-developed fruit, green fruit, immature fruit, and mature fruit.

Fig. 4. Predicted probabilities of *Botrytis cinerea* colonization of floral parts and fruit of red raspberry at each stage of flower and fruit development in 2015 and 2016. Bars with different letters indicate significant differences in colonization between organs within each developmental stage. Significance was estimated using the odds ratio with 95% confidence level, as described in the main text. Stages S1 and S2 were not included in this analysis due to low rates of colonization.

development of disease management tools even more challenging. We were unable to monitor levels of inoculum in the experimental field, but this is possibly a variable that should be monitored in future experiments. To evaluate the effectiveness of early fungicide sprays for control of *B. cinerea* that are routinely directed to flowering raspberry crops, it would be interesting to compare *B. cinerea* colonization of red raspberry flowers in fungicide-treated versus untreated fields.

The pathways of *B. cinerea* infection, infection courts, and the etiology of gray mold are still actively debated topics (Viret et al. 2004). A number of studies have connected *B. cinerea* infection of raspberry and other hosts with pollination (McClellan and Hewitt 1973; McNicol et al. 1985; Pezet and Pont 1986; Viret et al. 2004).

Pollination and fertilization of raspberry ovules results in the flower ovaries becoming part of each drupelet, with drupelets forming the aggregate fruit. As a fruit develops, other flower parts such as stamens and sepals senesce but remain attached at the base of the fruit. Senescent tissue is hypothesized to be a suitable substrate for colonization of a necrotrophic fungus such as *B. cinerea*. In our study, carpels had the greatest incidence of *B. cinerea* recovery at all developmental stages when plated on agar media compared with other flower/fruit component parts. Our results support previous findings of the role of carpels in infection of raspberry by *B. cinerea* (Dashwood and Fox 1988; McNicol et al. 1985; Williamson et al. 1987); however, our observed rates of colonization of stamens in flowers were lower than that observed by Dashwood and Fox (1988)

Fig. 5. Venn Diagram of *Botrytis cinerea* colonization of parts of red raspberry flowers and fruit in 2015. **A**, Stage S3 (open flowers); **B**, stage S4 (half-developed fruit); **C**, stage S5 (green fruit); **D**, stage S6 (immature fruit); and **E**, stage S7 (mature fruit). Each circle represents a different floral or fruit part. Number in a circle = percentage of “positive” samples colonized by *B. cinerea*. Overlapping areas show percentage of samples that had colonization of more than one part. Venn diagrams were created in R Studio version 1.0.136 using the package “VennDiagram” version 1.6.17. “Positive” samples = samples at each developmental stage colonized by *B. cinerea*. Stage S2 was not included in the analysis due to absence of *B. cinerea* colonization.

in their field experiment employing natural inoculum. As fruit developed in our study, other flower parts were increasingly colonized by *B. cinerea* and stamens became the second most frequently colonized part starting from stage S4. The frequency of samples with multiple component parts colonized by the fungus also increased with fruit development. We speculate that colonization of plant tissues adherent to raspberry fruit may be a potential source of external fruit infection later in the season. Similar observations were reported by McNicol et al. (1990) using artificial inoculation experiments of raspberry flowers and fruit. Dashwood and Fox (1988) also suggested that infected floral parts, such as stamens, could represent a significant source of inoculum for later

infections of raspberry fruit. The role of flower infections in gray mold development and potential external raspberry fruit infections needs to be investigated using molecular methods, such as tracking a pathogen through plant development using genetic markers, direct PCR or quantitative PCR (Fernandez et al. 2014; Gindro et al. 2005; Shaw et al. 2016).

Botrytis spp. require free water for conidial germination, germ tube growth, and penetration of plants; and the duration of the presence of moisture on plant tissue surfaces is widely used as a factor in modeling gray mold development and predicting epidemics (Williamson et al. 1995, 2007). In the field, disease development also may be affected by fluctuations in temperature

Fig. 6. Venn Diagram of *Botrytis cinerea* colonization of parts of red raspberry flowers and fruit in 2016. **A**, stage S2 (half-open flowers); **B**, stage S3 (open flowers); **C**, stage S4 (half-developed fruit); **D**, stage S5 (green fruit); **E**, stage S6 (immature fruit); and **F**, stage S7 (mature fruit). Each circle represents a different floral or fruit part. Number in a circle indicates percentage of “positive” samples colonized by *B. cinerea*. Overlapping areas show percentage of samples that had colonization of more than one part. Venn diagrams were created in R Studio version 1.0.136 using the package “VennDiagram” version 1.6.17. “Positive” samples = samples at each developmental stage colonized by *B. cinerea*.

Rain, mm
 Wetness, h
 Wetness, %
 RH, %
 CumRain, mm
 Temp, °C

Fig. 7. Environmental data during the red raspberry flower and fruit sampling season in 2015 and 2016 in Northwestern Washington to monitor colonization by *Botrytis cinerea*.

and RH. A rapid drop in RH and increase in temperature in the early morning causes drying and twisting of conidiophores of *B. cinerea* and the release of conidia into the air (Jarvis 1962). The importance of local environmental factors including air temperature, relative humidity, leaf wetness, and rainfall on gray mold epidemiology was previously reported by others (Blanco et al. 2006; Williamson et al.

1987; Xu et al. 2000, 2012). The present study also investigated the association of key environmental factors with *B. cinerea* colonization of raspberry.
The optimal temperature for *B. cinerea* infection is reported to be 20°C (Bulger et al. 1987; Jarvis 1962; Xu et al. 2000). Due to the nature of the experiments in this study, daily colonization of

Fig. 8. Percentage of *Botrytis cinerea* colonization of surface-disinfested samples of seven developmental stages of red raspberry in 2015 and 2016. Stages S1, S2, S3, S4, S5, S6, and S7 represent closed green buds, half-open flowers, open flowers, half-developed fruit, green fruit, immature fruit, and mature fruit, respectively. Heights of the bars in the histograms represent the percentage of samples that were positive for *B. cinerea* colonization at each stage and each sampling event.

raspberry flowers by *B. cinerea* was not monitored and, therefore, weekly averages of environmental conditions were used in the statistical analyses of the relationships between environmental conditions and *B. cinerea* colonization of flowers and fruit. Overall, the 2015 season was drier and hotter than the 2016 in NW Washington. Even though the weekly average air temperature did not differ significantly between 2015 and 2016, the 2015 season had significantly more days in which the maximum temperature was $>27^{\circ}\text{C}$, which is the upper limit for infection by *B. cinerea* (Xu et al. 2000). The 2015 season also had significantly less rain compared with 2016. Therefore, the significantly hotter and drier environmental conditions in 2015 may account, in part, for significantly lower incidences of *B. cinerea* recovery from raspberry flowers and fruit at all developmental stages throughout the season than in 2016.

Although 2015 was much drier than 2016 overall, there was more rain at the beginning of the 2015 season during flowering compared with the 2016 season, while in 2016 the cumulative level of rain was almost three times greater in the second part of the season compared with 2015. The earlier rain events in 2015 did not appear to increase *B. cinerea* colonization of raspberry flowers as the fungus was not recovered on any of the flower developmental stages during the first four weeks of sampling that year. This observation appears to support the hypothesis that flower infection is not the major route of gray mold development on raspberry in NW Washington. Similar correlations between high rainfall right before and during raspberry harvest and a high incidence of gray mold were reported by Jarvis (1964). Since mature red raspberry fruit is more susceptible to *B. cinerea* infection than flowers or green fruit (Jarvis 1962), rain events seem to be much more important in the latter part of the season and rainy weather before and during harvest may lead to greater gray mold incidence in raspberry fields than years with dry conditions during these periods of greater susceptibility to infection.

In conclusion, this study has provided novel insights into the potential pathways of infection that result in gray mold of raspberry under natural field conditions. Since the incidence of colonization of raspberry flowers by *B. cinerea* was consistently less than 15% during two growing seasons, we speculate that disease management strategies may be more effective if focused on protecting developing raspberry fruit rather than flowers. This study revealed no evidence that flowers are the exclusive or even the major infection route for development of gray mold of red raspberry in NW Washington. The relationship between *B. cinerea* infection of raspberry flowers and subsequent development of gray mold on the fruit could be investigated using molecular markers as an alternative test of the flower infection hypothesis. Our results also indicate that local environmental conditions play an important role in determining the risk of gray mold incidence and development and provides practical implications for the improvement of disease management practices in NW Washington. Average weekly minimum air temperature, average weekly night air temperature, cumulative rain, average weekly leaf wetness percentage, and duration of continuous leaf wetness $>90\%$ should be all monitored through the red raspberry season because all these factors were significantly correlated with *B. cinerea* colonization of raspberry in NW Washington over two years of study. Based on the results of this study we conclude that in western Washington rain is important for gray mold development, however if the season is dry, air temperature may become the limiting factor for *B. cinerea* to infect red raspberry, so monitoring air temperature and rainfall should be conducted simultaneously.

NW Washington produces approximately 80% of all processed red raspberry in the United States, and gray mold caused by *B. cinerea* is one of the major threats to the industry in this area. Disease management is based on full season fungicide application programs that start at 5 to 10% bloom and continue on a calendar basis throughout the raspberry season. Despite these intensive disease management programs, growers routinely experience fruit losses due to gray mold. The management programs consist of

average of five to seven fungicide applications per season, independent of disease pressure and infection risk. Such frequent fungicide applications are labor and cost-intensive and also have led to extensive fungicide resistance of pathogen populations to four of five fungicides registered for the control of gray mold in the U.S. PNW. Hence, there is a great need for improved disease management strategies that rely on disease biology rather than on calendar-timed sprays. We speculate that red raspberry growers in NW Washington may be able to decrease fungicide use substantially by eliminating early sprays targeted at protection of flowers. However, such a strategy would need to be verified using field-based efficacy studies conducted over multiple seasons and locations before being recommended and deployed on a regional scale. Eliminating or substituting early sprays could contribute to substantial cost reductions for growers and help reduce the risk of fungicide resistance development by *B. cinerea* by reducing overall selection pressure for fungicide resistance.

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