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# THE GROWTH RATE OF APPLE BITTER ROT LESION, CAUSED BY *COLLETOTRICHUM* SPP., IS AFFECTED BY TEMPERATURE, FUNGAL SPECIES, AND CULTIVAR

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**A B S T R A C T**

The growth and symptom development of two fungal species, *Colletotrichum siamense,* and *C. fioriniae* were examined using *in vitro* and *in planta* assays. In a plate assay, *C. siamense* grew fastest at 30 °C and at 25 °C, but *C. fioriniae* grew slower at 30 °C than at 25 °C. With apple inoculation assay, the mean lesion diameters at 30 °C were significantly larger ($P \leq 0.05$) than that at 20 °C on a cultivar Ida Red for both fungal species. On the other hand, the mean lesion diameter on a cultivar Golden Delicious was significantly larger at 20 °C than at 30 °C for both fungal species. Therefore, the rate of lesion development was influenced not only by fungal species and incubation temperature but also by apple cultivar. In this study, cultivar 'Ida red' was found to be more susceptible to *C. siamense* under warm environmental condition. Although both species were able to grow at 10 °C in the plate assay, no disease symptoms were developed at 10 °C with the inoculation assay. Although the numbers of cultivar and fungal species are small, to our knowledge, this is the first report to describe potential interactive effect among *Colletotrichum* species, temperature, and apple cultivar on the rate of bitter rot symptom development.

**Keywords**: *Colletotrichum siamense*, *C. fioriniae*, apple, bitter rot.

## INTRODUCTION

Bitter rot of apple (*Malus domestica* Borkh) is caused by multiple fungal species in the *Colletotrichum acutatum* and *C. gloeosporioides* species groups (Damm *et al.,* 2012; Weir *et al.,* 2012). The symptoms of bitter rot often appear as brown colored circular lesions on apple fruit surface that contains a group of the fungal fruiting body (acervuli) formed in concentric rings (Biggs and Miller 2001). When the infected fruit is cut open, "V"-shaped internal necrosis is often observed. There were studies on the susceptibility of apple cultivars (Biggs and Miller 2001; Jurick *et al.,* 2011), and infection process (Shane and Sutton 1981), but information on the difference between *Colletotrichum* species and on the rate of disease development on apple is limited.

Lesion expansion can be a significant component of the plant disease epidemic (Berger *et al.,* 1997). In some plant pathosystems, such as Phomopsis cane and leaf spot of grape, the pathogen, *Phomopsis viticola*, causes discrete lesions that remain constricted in size (Erincik *et al.,* 2003). On the other hand, in other pathosystems, the lesions continue to grow until much or all of the affected tissue becomes symptomatic. The potential consequences of lesion expansion are: disease severity can progress even though susceptible host tissue is no longer available for infection (Berger 1973); lesions can continuously expand even under less desirable environmental conditions for infection (Emge *et al.,* 1975); and the expanded lesion can provide substantially more area for the production of inoculum (Lannou *et al.,* 1994).

The objective of our research is to determine the effect of temperature, fungal species, and two apple cultivars on the development of bitter rot symptoms. The information obtained from this research will help us to determine factors affecting the rate of bitter rot symptom development, which can help decide fungicide application plans in the field.

## MATERIALS AND METHODS

**Preparation of fungal isolates:** Isolates of *C. fioriniae* (belongs to *C. acutatum* group, designated CTCH1A11A) and *C. siamense* (belongs to *C. gloeosporioides* group, designated ACAP1A11A) were grown on one-quarter strength Potato Dextrose Agar (PDA) amended with streptomycin and chloramphenicol (100 mg/ml each) for 10-14 days before each experiment. Hereafter, we refer this medium as the amended ¼ PDA. For each experimental run, mycelia were obtained from an independently grown plate. Species identification was made based on colony and spore morphology, and internal transcribed spacer (ITS) region sequence (Damm *et al.,* 2012; Weir *et al.,* 2012). Based on a BLAST search (Bonants *et al.,* 2013), a similarity score to *C. siamense* was 98.9% and to *C. fioriniae* was 100%. NCBI GenBank accession number for our *C. siamense* and *C. fioriniae* isolates are JX010245 and JQ948343, respectively.

**Fungal colony growth measurements:** A piece of media containing mycelium was harvested with a 5 mm diameter cork borer from *C. fioriniae* and *C. siamense* grown on the amended ¼ PDA and transferred onto the surface of PDA in a 90 mm Petri dish. Plates were incubated at 10 °C, 15 °C, 20 °C, 25 C, and 30 °C in a growth chamber (Thomas Scientific model 3759, Swedesboro, NJ), and the diameter of the mycelial growth was measured at 3, 5, 7 12, and 14 days, and a value of the measured diameter minus 5 mm, which was the size of the original piece, was recorded. The experiment was conducted on October 2014. For each experimental run, there were five internal replications, and there were two experimental runs.

**Lesion size experiment:** Visually clean apple fruits were collected from an experimental orchard located at AHS Jr. Agricultural Research and Extension Center (AHS AREC) in Winchester, Virginia, USA on October 2013. Two cultivars 'Ida Red' and 'Golden Delicious' were used. No chemical application was made for post-harvest diseases. Harvested apples were placed on a preformed cardboard sheet where each apple is physically separated from others. The cardboard sheet with apples was then placed into a plastic incubation chamber. The incubation chamber with apples was placed in a designated growth chamber two days in advance of the experiment so that the temperature of the fruit is close to that of the environment.

Before inoculation, the apple surface was disinfected with 70% EtOH. EtOH solution was sprayed until run-off with a hand atomizer, then wiped with a clean paper towel. A tuft of mycelium from the surface of the media was harvested using an autoclaved toothpick. Then the mycelium was inserted into an apple by stabbing. The depth of the wound was approximately 1 cm. We incubated apples under three temperature conditions, 10 °C, 20 °C, and 30 °C. Light conditions were set to 16 hours of light and 8 hours of darkness each day. A temperature/relative humidity sensor was placed inside the plastic chamber to monitor the environmental condition to monitor the condition in the chamber. There were five apples per isolate per experimental run, and the experiment was repeated three times. Assessment of disease was made visually every other day until 12 days after inoculation. When a symptom developed, the diameter of the lesion was recorded.

**Statistical analysis:** A standard least-square model was used to determine the effect of temperature and fungal species on colony growth rate on PDA. A regression analysis was conducted to determine the slope and intercept for colony growth over time. Analysis of variance (ANOVA) was conducted to determine the effect of temperature, fungal species, and apple cultivar on the mean diameter of the lesion at 12 days after inoculation. A standard least-square model was used. When a factor or interaction of factors was significant, multiple comparisons were made using Tukey-Kramer adjustment. Analyses were conducted using JMP Pro (version 14, SAS Institute, Cary, NC).

## RESULTS

**Effect of temperature on fungal colony growth:** The effect of both fungal species and temperature were significantly affected ($P \leq 0.05$) the colony diameter measured over the course of 14 days (Table 1). *C. siamense* resulted in significantly larger mean colony diameter (36.6 mm) than *C. fioriniae* (30.1 mm). Moreover, there was a significant three-way interaction among fungal species, temperature and day after transfer, which indicated the rate of increase in the colony diameter over time differ by fungal species and temperature.

*C. siamense* generally grew faster than *C. fioriniae*. At day 3, *C. siamense* cultures grew at 25 °C and 30 °C resulted in the mean diameter above 25 mm (Figure 1). At these two temperatures, the colony diameter reached close to the limit (85 mm) by the seventh day after the transfer. By day 12, the mean colony diameter for the culture grew at 20 °C also reached 85 mm. On the other hand, not all *C. fioriniae* cultures reached the edge of the Petri plate, even

at 25 °C, which was the temperature with the fastest growth (i.e., the largest slope coefficient). Also, *C. fioriniae* grew at 30 °C showed slower growth (i.e., lower slope coefficient) than growth rate at 20 °C or 25 °C. Both species grew slower at 10 °C and 15 °C. At 10 °C, the final mean diameter at 14 days after the transfer was 20 mm and 16.3 mm for *C. fioriniae* and *C. siamense*, respectively.

Table 1. The results of a standard least-squares model to determine the effect of species and temperature on the colony diameter on PDA measured over the course of 14 days.

| Source | *F* Ratio | *P* > *F* |
|---|---|---|
| Species | 1002.9 | < 0.01 |
| Temperature | 2278.2 | < 0.01 |
| Day after transfer | 9009.9 | < 0.01 |
| Day after transfer*species | 126.1 | < 0.01 |
| Day after transfer*temperature | 379.8 | < 0.01 |
| Species*temperature | 244.4 | < 0.01 |
| Day after transfer*species*temperature | 18.9 | < 0.01 |

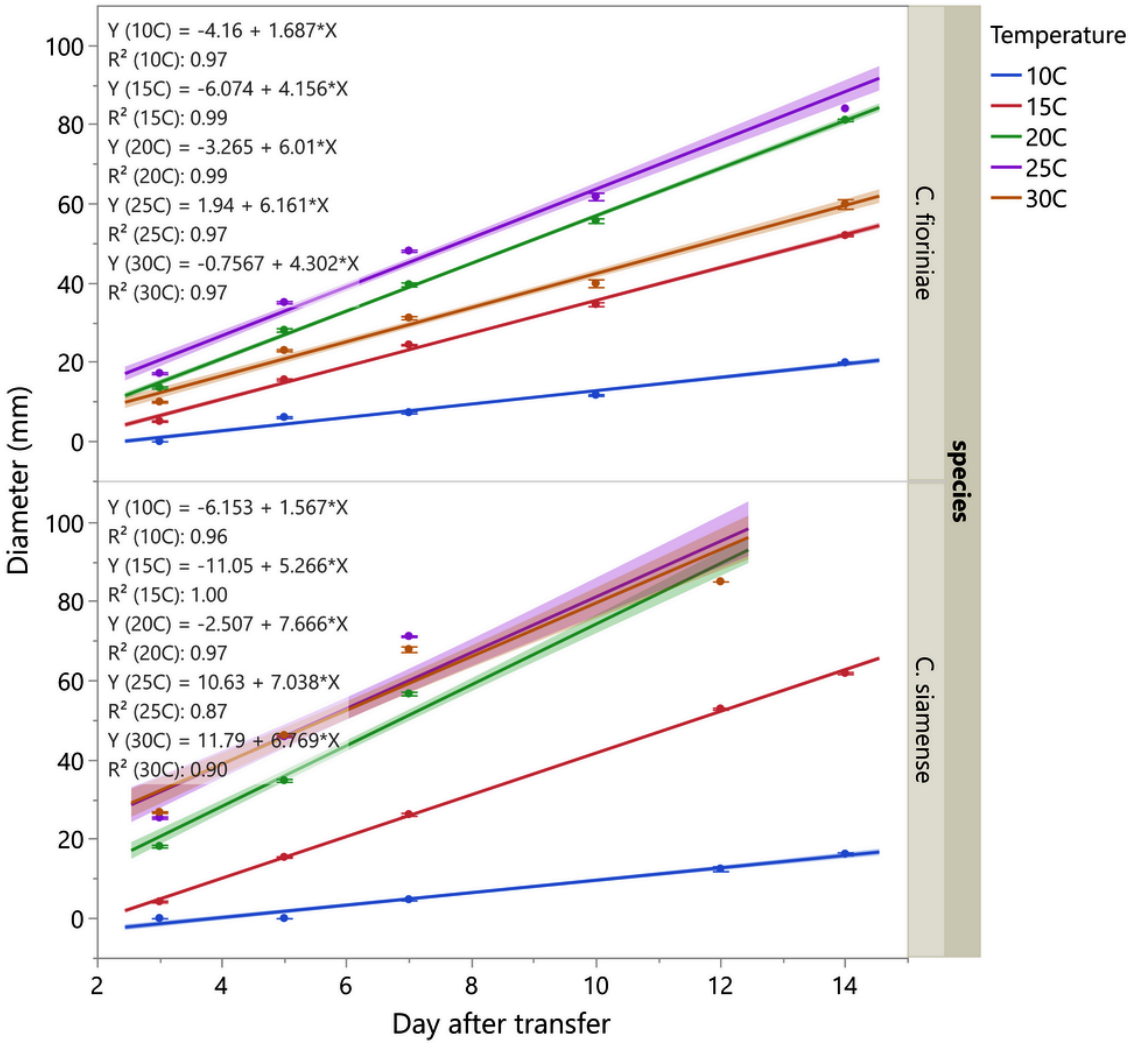


Figure 1. Regression lines and a 95% confident interval of colony diameter by fungal species and temperature over time (day after transfer). The error bars represent standard errors. Equations and R-square values for each line of fit are presented. The maximum diameter is 85 mm.

**Effect of cultivar, fungal species, and temperature on lesion size:** At day 12 of disease assessment, mean lesion diameters varied from 0 mm to 67 mm. At 10 °C, there was no disease development (Figure 2). Overall, *C. siamense* resulted in larger lesions than *C. fioriniae*. The results from ANOVA showed that there was a three-way interaction among temperature, fungal species, and apple cultivar ($P < 0.01$) impacted on the mean diameter of the lesion (Table 2).

In other words, the lesion size of two tested fungal species was affected by temperature and cultivars, and results were not consistent over tested temperatures and cultivars. With *C. siamense*, the mean lesion diameter was the greatest on Ida Red incubated at 30 °C, which was significantly larger ($P \leq 0.05$) than on Golden Delicious at the same temperature (Figure 2). On the other hand, when incubated at 20 °C, there was no significant difference between the two cultivars ($P > 0.05$). The mean lesion diameter with *C. fioriniae* incubated at 30 °C resulted in a significantly larger diameter on Ida Red than that with Golden Delicious, while at 20 °C, the mean diameter on Golden Delicious was significantly larger than that on Ida Red. In general, the mean lesion diameter with *C. siamense* resulted in the same or larger than that with *C. fioriniae*.

Table 2. Analysis of variance table to show the effect of fungal species, apple cultivar, temperature and combination of factors based on the standard least squares (JMP Pro 14).

| Source of variation | *F* Ratio | *P* > *F* |
|---|---|---|
| Species | 89.0 | < 0.01 |
| Cultivar | 7.4 | 0.01 |
| Temperature | 130.5 | < 0.01 |
| Species*cultivar | 19.9 | < 0.01 |
| Temperature*cultivar | 24.0 | < 0.01 |
| Temperature*cultivar | 61.8 | < 0.01 |
| Temperature*species*cultivar | 7.0 | < 0.01 |

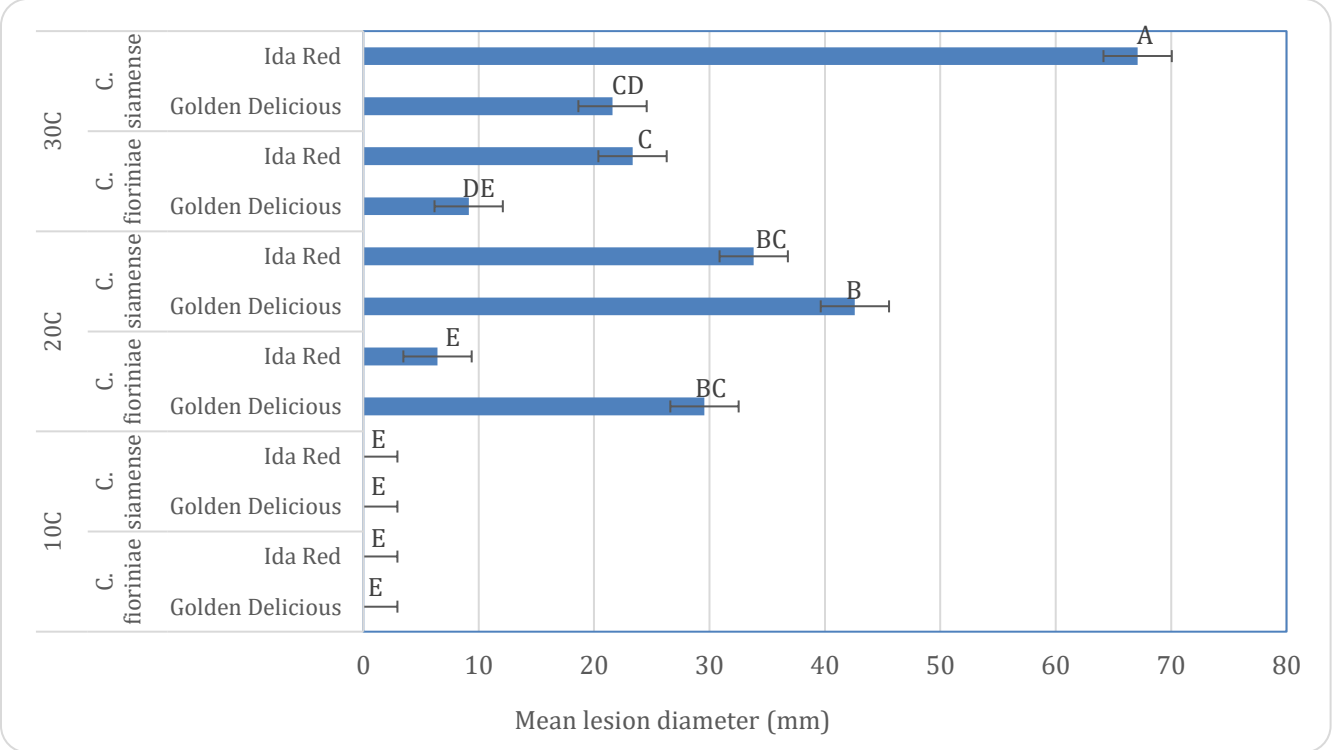


Figure. 2 Mean lesion diameters in mm at assessment day 12 by temperature, fungal species, and apple cultivar. Error bars represent standard errors. Different letters between two bars indicate a significant difference ($P \leq 0.05$) based on Tukey-Kramer multiple comparisons.

**DISCUSSION**

This study compared the growth and symptom development of two fungal species, *Colletotrichum siamense* and *C. fioriniae* using *in vitro* and *in planta* assays. Although there were several studies documented the effect of temperature and other growing conditions on the development of disease caused by *Colletotrichum* species (Brown 1975; Fitzell *et al.,* 1984; Wilson *et al.,* 1990), the information is limited for recently classified species such as *C. siamense* and *C. fioriniae* used in this study. These two species are associated with apple bitter rot in recent studies in Kentucky, USA and Argentina (Fernandez *et al.,* 2018; Munir *et al.,* 2016). In general, *C. siamense* tends to grow faster than *C. fioriniae* in the plate assay, and that seemed to be the same in the apple inoculation assay. The more aggressiveness nature of *C. siamense* is also discussed in the Kentucky study (Munir *et al.,* 2016). Recently, two studies from Belgium described differences of pathogenicity among different *Colletotrichum* species (Grammen *et al.,* 2019) and cultivar susceptibility comparisons (Grammen *et al.,* 2018). These studies documented variable pathogenicity and cultivar susceptibilities, which matches results from this study.

Both species grew fast at 25 °C with the plate assay, and *C. siamense* grew as fast at 30 °C as at 25 °C, but *C. fioriniae* grew slower at 30 °C, indicating that *C. siamense* prefers higher temperature than *C. fioriniae*. On the other hand, the effect of temperature in the apple inoculation assay was not as clear as the plate assay. For both fungal species, the mean lesion diameters at 30 °C were significantly larger ($P \leq 0.05$) than that at 20 °C on Ida Red. On the other hand, the mean lesion diameter on Golden Delicious was significantly larger at 20 °C than at 30 °C for both fungal species on the contrary to the results of the plate assay. Therefore, the rate of lesion development was affected by not only by fungal species and incubation temperature but also by apple cultivar. Although the numbers of cultivar and fungal species are small, to our knowledge, this is the first report to describe potential interactive effect among *Colletotrichum* species, temperature, and apple cultivar on the rate of bitter rot symptom development. Differences in the production of volatile compounds such as ethylene and alcohol (Pentzer and Heinze 1954) or the rate of decomposition between cultivars may contribute to the difference in the symptom development, but further research is required.

In previous studies where fruits were wounded and inoculated, Golden Delicious tended to show larger lesion than other tested cultivars; however, these studies did not include Ida Red (Biggs and Miller 2001; Munir *et al.,* 2016). A post-harvest study with Ida Red indicated bitter rot was one of the common diseases they found (Sever 2012); however, we were not able to find the relative susceptibility of Ida Red to bitter rot, with an exception of a very recent study where the inoculation test was conducted in a fixed temperature (Grammen *et al.,* 2019a). Thus, it is important to note that Ida Red is probably more susceptible to bitter rot than Golden Delicious at a warmer condition.

Interestingly, although both species were able to grow at 10 °C in the plate assay, no disease symptoms were developed at 10 °C with the inoculation assay. Considering the inoculation method, where we artificially wound the surface of apple fruit, the lack of symptom development indicates that even if there is an infection, symptom development most likely does not occur at a low-temperature condition. This may add to the difficulty of detecting bitter rot infected apple fruits at the time of harvest.

In summary, our results showed that *Colletotrichum* species composition, apple cultivar, and environmental condition can have an impact on the rate of bitter rot disease development. This information can help growers prioritize their bitter rot management based on these criteria. In order to address this complex disease effectively, *Colletotrichum* species identification, cultivar selection, and monitoring of environmental condition has to be combined. For example, if an Ida Red orchard is located in a warm region, and if there is a history of *C. siamense*, more attention to bitter rot management is required.

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