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Difference in ergosterol concentrations extracted from demethylation inhibitor resistant and susceptible strains of *Monilinia fructicola*: understanding the 'Mona' element

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Abstract
Monilinia fructicola causes disease on peaches and nectarines, and is an economic concern in the South Eastern United States. This experiment was designed to try and understand the mechanisms that allow *M. fructicola* to be resistant to Demethylation Inhibitor (DMI) fungicides, which is an interest among agronomists. Isolates of *M. fructicola* were analyzed with High Performance Liquid Chromatography to measure differences in ergosterol concentration between selected groups of isolates. This was done to determine if *M. fructicola* is using an alternate sterol other than ergosterol as the final end product of the sterol biosynthesis pathway, or is using a different enzyme other than 14-α demethylase, to synthesize ergosterol. This experiment showed differences in ergosterol concentration between the groups of isolates that were analyzed. The three groups analyzed in this study included isolates without the 'Mona' element while susceptible to DMI fungicides, isolates with the 'Mona' element and resistant to DMI fungicides, isolates with the 'Mona' element while susceptible to DMI fungicides. Statistical analysis revealed significant differences between the three groups of isolates, which suggesting that *M. fructicola* uses a different enzyme, other than 14-α demethylase, to synthesize ergosterol.

Keywords: DMI fungicides, Ergosterol, *Monilina fructicola*, Resistance

تفاوت در غلظت ارگوسترول استخراج شده از استرین‌های مقاوم و حساس گونه *Monilinia fructicola* به مهار کننده دمتیلاسیون: درک عنصر "مونا"

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چکیده
Monilinia fructicola باعث بیماری در انواع شلیل و هلو شده و یک نگرانی اقتصادی در جنوب شرقی ایالات متحده محسوب می‌گردد. این تحقیق به‌منظور تلاش برای درک مکانیسم‌هایی که به *M. fructicola* این توانایی را می‌دهد تا در برابر قارچ‌کش‌های بازدارنده دمتیلاسیون (DMI) مقاوم باشد، طراحی گردید. جدایه‌های *M. fructicola* با کروماتوگرافی مایع با کارایی بالا برای اندازه‌گیری میزان تفاوت غلظت ارگوسترول بین گروه‌های منتخب جدایه‌ها، مورد بررسی قرار گرفت. این امر برای تعیین اینکه آیا *M. fructicola* از استرول دیگری به غیر از ارگوسترول به عنوان محصول نهایی مسیر بیوسنتز استرول استفاده می‌کند، یا از آنزیم متفاوتی به غیر از ۱۴- آلفا دمتیلاز برای سنتز ارگوسترول استفاده می‌کند، انجام شد. این آزمایش تفاوت بین غلظت ارگوسترول در میان گروه‌های جدایه‌هایی که مورد بررسی قرار گرفتند را نشان داد. سه گروه از جدایه‌ها در این تحقیق مورد تجزیه و تحلیل قرار گرفتند که گروه اول شامل جدایه‌های فاقد عنصر "مونا" و حساس به قارچ‌کش‌های DMI، گروه دوم شامل جدایه‌های دارای عنصر "مونا" و مقاوم در برابر قارچ‌کش‌های DMI، و گروه سوم شامل جدایه‌های دارای عنصر "مونا" و حساس به قارچ کش‌های DMI بودند. نتایج حاصل از تجزیه و تحلیل آماری تفاوت‌های معنی‌داری را بین سه گروه از جدایه‌ها نشان داد، که نشان می‌دهد *M. fructicola* از آنزیم متفاوتی به غیر از ۱۴- آلفا دمیتیلاز، برای سنتز ارگوسترول استفاده می‌کند.

کلمات کلیدی: قارچ‌کش‌های بازدارنده دمتیلاسیون، ارگوسترول، *Monilina fructicola*، مقاومت

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Introduction

Monilinia fructicola [Winter], the causal agent of brown rot and blossom blight on nectarines and peaches, is of major economic concern in the South Eastern United States (Dai *et al*. 2003; Schnabel & Bryson 2004). Nectarines and peaches are both members of the same species, *Prunus persica* [Batsch], which are one of the most economically important members of the *Rosaceae* family (Eldem *et al*. 2012). *Prunus persica* are a model organism for plant research and are a delicacy during the summer months in the eastern United States (Eldem *et al.* 2012). *Monilinia fructicola* has been shown to infect peaches and nectarines at various time during the growing season, including blossom emergence, pre-harvest, as well as post-harvest of fruit (Chen *et al.* 2012, Chen *et al.* 2013a; Chen *et al.* 2013b; Luo *et al.* 2008; Schnabel & Dai 2004). In conventional fruit production systems, *M. fructicola* is controlled by weekly spraying of fungicides from various chemical classes.

Fungicide resistance to Benzimidazoles (BZI) started to appear in the early 1980's (Chen *et al*. 2012; Chen *et al.* 2013a; Chen *et al*. 2013b; Luo *et al.* 2008; Schnabel & Dai 2004), and since then, Demethylation Inhibitor (DMI) fungicides have been routinely used for blossom blight and brown rot control (Chen *et al.* 2012; Chen *et al*. 2013a; Chen *et al.* 2013b; Luo *et al.* 2008; Schnabel & Dai 2004). The most commonly used DMI fungicides for control of *M. fructicola* are Propiconazole©, Fenbuconazole©, and Tebuconazole© (Chen *et al.* 2012; Chen *et al.* 2013; Chen *et al.* 2013; Luo *et al.* 2008; Schnabel & Dai 2004). The mode of action of DMI fungicides to control *M. fructicola* is that they bind to the sterol 14α-demethylase, where they inhibit sterol biosynthesis in fungal cell walls because 14-αdemethylase cannot demethylate a precursor to ergosterol, thus halting growth of the fungi (Schnabel & Bryson 2004). *M. fructicola* can develop reduced sensitivity to DMI fungicides after repeated exposure in the field, and can occur after as few as 12 applications over 3 years (Schnabel & Bryson 2004). Resistance can arise from mutations in the cytochrome p450 14-αdemethylase (*CYP51*) gene, over expression of (*CYP51*) genes, or by other unknown means (Luo *et al.* 2008, Schnabel & Dai 2004; Schnabel & Jones 2001).

The isolates for this experiment were chosen based upon whether they contained or did not contain a promoter region named 'Mona', which is upstream of the cytochrome p450 14-αdemethylase (*CYP51*) gene, and has been shown to confer fungicide resistance (Luo *et al*. 2008; Schnabel & Dai 2004; Schnabel & Jones 2001). Like many other promoting sequences, 'Mona' is located in the 5' direction before the DNA region that coded for 14α-demethylase (Chen *et al.* 2013; Luo *et al.* 2008). The number of isolates was limited to two of each of the following categories: without 'Mona' element and resistant to DMI fungicides (MDBBC-6, MDBBC-9), with 'Mona' element and resistant to DMI fungicides (SCTIT-1, SCTIT-2), and without 'Mona' element but susceptible to DMI fungicides (SCMD1-7-11, SCMD2-12) (Chen *et al.* 2012; Chen *et al.* 2013). The fourth category of isolates are strains of *M. fructicola* that contain the 'Mona' element, but are susceptible to DMI fungicides. Other laboratories have collected these isolates, but the Schnabel Lab did not have isolates on hand that contain the 'Mona' element and are susceptible to DMI fungicides, so these isolates will be omitted from the study (Chen *et al.* 2013). Two isolates of each category were chosen due to limited available space in Clemson University's Multi-User Analytical Laboratory, and were grown in technical

replicates of ten, in order to make this study more robust.

This project is aimed at determining the physiological mechanisms that allow *M. fructicola* to become resistant to DMI fungicides. By comparing the total ergosterol levels in various strains of *M. fructicola,* the researcher will be able to determine if *M. fructicola* is using an alternate sterol, other than ergosterol, as the final end product of the sterol biosynthesis pathway, or if a different enzyme, other than 14α demethylase, is being utilized within the ergosterol biosynthesis pathway, to synthesize ergosterol (Schnabel & Jones 2001). It is well known that ergosterol is the main fungal sterol used to build rigidity within the plasma membrane and cell wall. This difference in ergosterol concentration will be deduced by comparing the peak's area produced from high performance liquid chromatography (HPLC) analysis, to a standard curve produced from a control concentration of ergosterol. If there were a difference in peak area, this would suggest that ergosterol biosynthesis is being affected by the presence or absence of the 'Mona' element, and another sterol is probably being synthesized instead of ergosterol, or an alternative enzyme is being utilized to create ergosterol (Chen *et al.* 2012; Chen *et al*. 2013a; Luo *et al.* 2008b; Schnabel & Dai 2008; Schnabel & Jones 2001). If there were no difference in peak area, this would mean that *M. fructicola* is probably using an alternate enzyme, within the ergosterol biosynthesis pathway, to produce an intermediary product, which then goes onto make ergosterol in the presence of a DMI fungicide. This study is designed to provide information in the form of data to expand the field's further understanding of the mechanism for DMI resistance in *M. fructicola.*

Materials & Methods

Mycelial Growth

Briefly, isolates were removed from -80 ˚C freezers, and grown on PDA media for one week. Then, ten replicates of each isolate were cut from the PDA plates, and grown in potato dextrose broth (PDB) media at 22 ± 2 ˚C for seven days on multiple shakers. The resulting mycelial mass was filtered with a vacuum filter, then washed once with distilled water, and the filter paper containing the mycelia was placed in a desiccator with DryRite©, until thoroughly dried. The mycelium from the technical replicates was then removed from the filter paper, homogenized, and 0.1g of each sample was added to its own glass screw-cap vial.

Total Ergosterol Extraction:

Total ergosterol was extracted by using methods of the Arthington-Skaggs method, as well as parts of the Alcazar-Fuoli method (Alcazar-Fuoliet *et al.* 2008; Arthington-Skaggs *et al.* 2000; Arthington-Skaggs *et al.* 1999). Further method development was created by parameters found in corresponding literature (Griffiths *et al.* 2003; Muimba-Kankolongo & Bergstrom 2010; Nes & Nichols 2006; Pinto *et al.* 2011; Shirane *et al.* 1996; Tangni *et al.* 2006; Yuan *et al.* 2008). The homogenized fungal mass in screw cap vials was mixed with 3 ml of 25 % alcoholic potassium hydroxide solution (25 g KOH in 36 ml of distilled water, brought up to 100 ml with 100 % ethanol). Mixture was homogenized for one minute on a vortex with glass beads than in a bead homogenizer. The cellular suspension was incubated for one hour at 80 ˚C. The suspension was cooled to 22 ˚C. Once cooled, 1 ml of distilled water and 3 ml of N-heptane (hexane) was added to the tube. This solution was vortexed for three minutes, to ensure all of the hexane was allowed to contact the sample. After homogenization, the upper layer (heptane / hexane layer) was collected and transferred to glass

screw cap vials. The volume of heptane (hexane) that was recovered from this process was recorded. The sample was dried under a nitrogen stream for 30 minutes, until the sample has condensed from its original volume down to dry. The condensed volume was brought back up to 1ml with 50 % methanol, by adding 500 ul of 100 % methanol, then adding 500 ul of distilled water. The samples were now ready for analysis with high performance liquid chromatography.

Creating a Standard Curve for Analysis

Stock solutions of ergosterol were obtained from Sigma Aldrich©, at a concentration of crystalline purity. A stock solution of 1000 μg/μl was made, and the stock solution was diluted to a concentration of 0.1 ,0.5, 2.5, 12.5 μg/μl, and injected into the HPLC to use as a standard in which the various treatment samples were compared (Figure 3).

The samples were analyzed with a Shimadzu© HPLC, with a UV-Photo Diode Array detector with both tungsten and a deuterium lamp turned on. The samples were run with a 100 % methanol gradient, at a flow rate of 1 ml/minute. A C-18 reverse phase column was used due to the non-polar nature of ergosterol. All data was analyzed at a wavelength of 282 nm. Sample peaks were read, and compared to the standard curve for quantification of data.

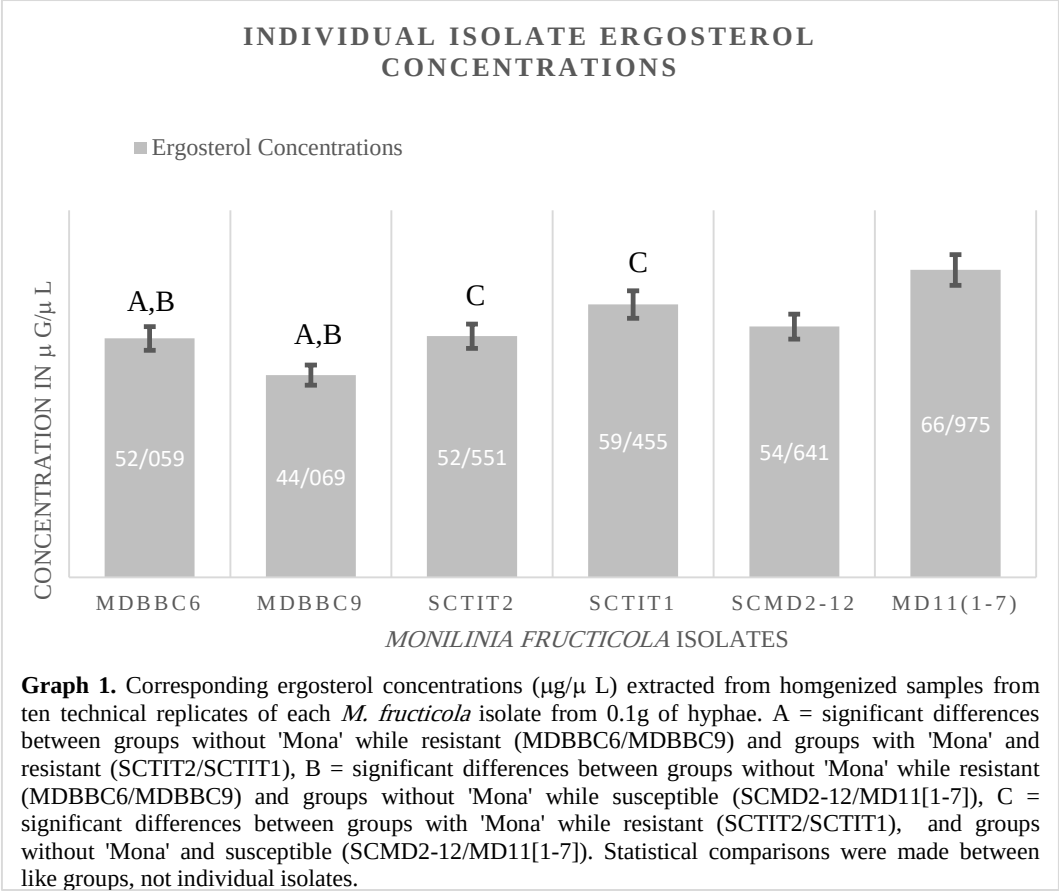
Statistical Analysis

Samples were analyzed with the Microsoft© Excel© Data Analysis package, and data was recorded. Samples were analyzed with a two tailed T-Test (assuming equal variance) to see if the concentrations of ergosterol were different between samples containing the 'Mona' element and samples not containing the 'Mona' element. The samples were further analyzed with a two tailed T-Test (for means) to see if the mean concentration of ergosterol were different or similar between the treatment groups. An ANOVA was performed in order to see similarities and differences between all three groups.

Table 1: The *Monilinia fructicola* isolates and corresponding ergosterol concentrations used for this experiment.

Sample	**Mona Element**	**Resistance to DMI Fungicide**	**Total Ergosterol per 0.1 gram**
MDBBC6	0	1	52.059
MDBBC9	0	1	44.069
SCTIT2	1	1	52.551
SCTIT1	1	1	59.455
SCMD2-12	0	0	54.641
MD11(1-7)	0	0	66.975

Presences of 'Mona' element designated 1 for present and 0 for absent. Resistance to DMI is denoted as 1 for resistant and 0 for susceptible. Total ergosterol concentration is expressed in (μg/μl).



Graph 1. Corresponding ergosterol concentrations (µg/µ L) extracted from homgenized samples from ten technical replicates of each *M. fructicola* isolate from 0.1g of hyphae. A = significant differences between groups without 'Mona' while resistant (MDBBC6/MDBBC9) and groups with 'Mona' and resistant (SCTIT2/SCTIT1), B = significant differences between groups without 'Mona' while resistant (MDBBC6/MDBBC9) and groups without 'Mona' while susceptible (SCMD2-12/MD11[1-7]), C = significant differences between groups with 'Mona' while resistant (SCTIT2/SCTIT1), and groups without 'Mona' and susceptible (SCMD2-12/MD11[1-7]). Statistical comparisons were made between like groups, not individual isolates.

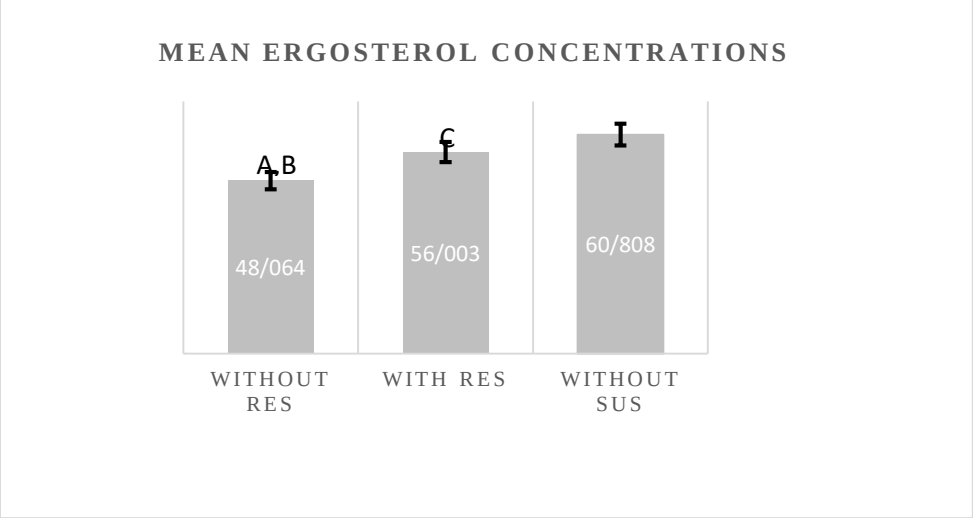


Figure 2. Grouped average total ergosterol concentration in (µg/µL) extracted from 0.1 g of hyphae. Without res = without 'Mona' element and resistant, with res = with 'Mona' element and reistant, without sus = without 'Mona' element and suseptable, A = significant differences between groups without 'Mona' while resistant and groups with 'Mona' and resistant, B = significant differences between groups without 'Mona' while resistant and groups without 'Mona' while susceptible, C = significant differences between groups with 'Mona' while resistant and groups without 'Mona' and susceptible.

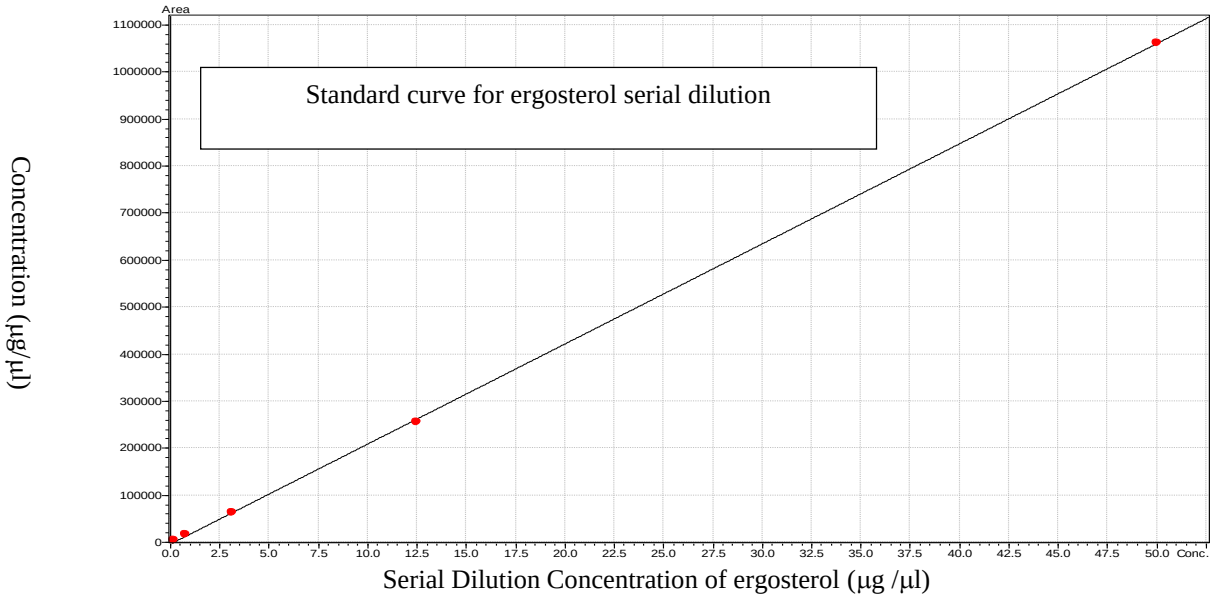


Figure 3. Standard curve for logistic ergosterol serial dilution. 0.1, 0.5, 2.5, 12.5 µg/µL.

Results

Table 1 shows all the data collected for this experiment and is the result of homogenizing samples from ten technical replicates of each isolate used in this study.

Figure 1 represents the total ergosterol in each sample with the isolate name on the x-axis and the concentration of ergosterol on the y-axis. A 95 % confidence interval is represented the by error bars for reach isolate. Figure 2 shows the total ergosterol averaged between isolates that share the same genotypic make-up. The error bars for each group represent a 95 % confidence interval. These error bars are used simply to predict where the researcher suspects the range of total ergosterol if replications were made for this experiment. Figure 3 represents the standard curve of the dilutions prepared from 1000 µg/µL stock ergosterol. A stock solution of 1000 µg/µL was made, and the stock solution was diluted to a concentration of 0.1, 0.5, 2.5, 12.5 µg/µL. The standard curve (Graph 3) had an R^2 value of 0.999, which shows a very strong correlation between the diluted samples of ergosterol, which means the standard curve was performed with accuracy and precision.

The average ergosterol for isolates without the 'Mona' element and resistant to DMI fungicides is 48.064 µg/µL in 0.1 gram of mycelia (Figure 2). The average ergosterol concentration for isolates with the 'Mona' element and resistant to DMI fungicides was 56.003 µg/µL in 0.1 gram of mycelia (Graph 2). The average ergosterol concentration for isolates without the 'Mona' element and susceptible to DMI fungicides was 60.808 µg/µL in 0.1 gram of mycelia (Graph 2). When all of concentrations mentioned previously were averaged, the mean total ergosterol for all of the samples in *M. fructicola* was 54.9583 µg/µL in 0.1 gram of mycelia.

After performing a two tailed T-Test (assuming equal variance) between isolates without the 'Mona' element while resistant to DMI fungicides compared to isolates with the 'Mona' element and resistant to DMI fungicides, the *p*-value was 0.0069, which is significantly different (supplementary table 1). After

performing a two tailed T-Test (assuming equal variance) between isolates without the 'Mona' element while resistant to DMI fungicides compared to isolate without the 'Mona' element and susceptible to DMI fungicides, the *p*-value was 0.011, which is significantly different (supplementary table 2). After performing a two tailed T-Test (assuming equal variance) between isolates with the 'Mona' element while resistant to DMI fungicides compared to isolates without the 'Mona' element and susceptible to DMI fungicides, the *p*-value was 0.013, which is significantly different (supplementary table 3).

Statistical ANOVA test revealed significant differences between the groups of isolates with a *p*-value of 8.409×10^{-11}, suggesting that at least one of the three groups differ from each other (supplementary table 4). This is consistent with the *p*-values generated from the two-tailed T-Tests mentioned previously.

Discussion

Monilinia fructicola causes multiple disease symptoms on peaches /nectarines, and is a major economic problem in conventional fruit productions systems in the South Eastern United States (Schnabel & Bryson 2004). Nectarines as well as peaches, *P. persica*, are an economically important members of the *Rosaceae* family, and have been used as a model organism for plant research (Eldem *et al.* 2012). In conventional fruit production systems, *M. fructicola* is controlled by weekly spraying of fungicides from various chemical classes, and reduced sensitivity to fungicides, in particularly DMI fungicides, after repeated exposure in the field, occurs regularly. It is important to understand the biochemical processes that confer resistance so alternative measures can be implemented, which allow farmers to adequately control *M. fructicola* in the fields (Schnabel & Bryson 2004).

After completion of this experiment, a baseline for total ergosterol from *M. fructicola* was achieved by liquid-liquid extraction and analysis of the samples with HPLC. The mean total ergosterol concentration for 0.1 gram of dry *M. fructicola* mycelia was 54.9583 μg/μL. This baseline can be used for novel experiments and by future researchers interested in *M. fructicola* cell wall metabolism and composition.

After statistical analysis, results showed significant differences between the three different isolate groups of *M. fructicola*, both in the T-Tests (assuming equal variance), and the ANOVA. These results suggest that *M. fructicola* uses an alternate enzyme within the sterol biosynthesis pathway, other than 14α-demethylase, which is being utilized within the ergosterol biosynthesis pathway, to synthesize ergosterol precursors (Schnabel & Jones 2001). Since the data was generated from ten technical replicates and consolidated for each isolate, statistical analysis should be assumed to be very accurate in this situation.

Analysis of the three groups of isolates used in this experiment provided results that were intriguing and unexpected. It was expected that isolates without the 'Mona' element and susceptible to DMI fungicides would produce the least amount of ergosterol, while the other isolates would produce more ergosterol. This is due to the fact that DMI resistant isolates often have the 'Mona' element promoter, which increases the production of 14α-demethylase, and thus the fungi produce more ergosterol (Luo *et al.* 2008).

The group of isolates that produced the least amount of ergosterol was the group without the 'Mona' element while resistant to DMI fungicides. This was expected due to the fact that they lacked the 'Mona' element, which is a promoter region that

promotes the transcription of 14α-demethylase, which is used in the biosynthesis pathway of ergosterol. The fact that this group of isolates produces the least amount of ergosterol and were resistant to DMI fungicides only supports what would be logical in this situation, suggesting that resistant strains are using another enzyme or enzyme precursor, in the ergosterol sterol synthesis pathway. Another explanation is their resistance arrived from a mutation in the CYP51 gene or by other means. The group isolates that produced the most ergosterol was the group that did not have the 'Mona' element, while was susceptible to DMI fungicides. This group of isolates produced concentrations that were higher than to be expected in this study, because the researcher expected these isolates to produce less ergosterol. Since they do not have the promoter sequence to make 14α-demethylase, which is a precursor of ergosterol synthesis, which should result in less ergosterol being produced. These results were unexpected and opposite of what was predicted. Perhaps these groups of isolates are the 'wild type', and the concentrations they are producing are standard for *M. fructicola.* Another explanation for these results in the isolates without the 'Mona' element while susceptible to DMI fungicides is that they are using other enzymes in the ergosterol synthesis pathway that bolster the production of ergosterol, while using up the cells energy to reproduce, thus halting the growth of this fungi. The final explanation of why the ergosterol concentrations were not as expected is that perhaps *M. fructicola* is using a different promoter within this biosynthesis pathway (Villani and Cox 2011). The group of isolates which produced the middle amount of ergosterol were cultures that had the 'Mona' element and were resistant to DMI fungicides. This is somewhat unexpected due to the fact that they contained the 'Mona' promoter sequence, which suggests that they would produce more ergosterol as an end result, but this is not the case.

Completion of this experiment has left room for future research questions which could help researchers better understand DMI fungicide resistance. This experiment could be expanded by having the fourth genotypic group that was omitted from this study, the isolates with the 'Mona' element but susceptible to DMI fungicides, to serve as another treatment for the study. The only reason why these isolates were omitted from this study was because they were not directly available in the Schnabel laboratory during the time of experimentation, but they would make for an excellent follow up study to help elucidate the means of DMI fungicide resistance. It would be predicated, based on the results from this study, that these isolates would have a moderate amount of ergosterol production compared to the other groups of isolates. Future research would be needed to confirm these predictions.

Conclusion

In conclusion, this experiment was designed to try and understand the mechanisms that allow *Monilinia fructicola* to be resistant to DMI fungicides. One of the most important aspects of this experiment is to determine that the mean total ergosterol concentration for *M. fructicola* was (54.9582 μg/μL) per 0.1 gram of mycelium. This experiment showed a significant difference in ergosterol concentration between the three groups of isolates; isolates without the 'Mona' element while resistant to DMI fungicides compared to isolates with the 'Mona' element and resistant to DMI fungicides, isolates without the 'Mona' element while resistant to DMI fungicides compared to isolate without the 'Mona' element and susceptible to DMI fungicides, isolates with the 'Mona' element while

resistant to DMI fungicides compared to isolates without the 'Mona' element and susceptible to DMI fungicides. An ANOVA test revealed significant differences between the three groups of isolates, suggesting that at least one of the three groups differ from each other. This suggest that *M. fructicola* is using an alternate sterol precursor, as the intermediate product of the sterol biosynthesis pathway, or is using a different enzyme, other than 14α-demethylase, to synthesize ergosterol.

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