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Differentiation of Fungal Phytopathogens by FT-IR and MALDI-TOF MS

Curtis Muldrow Atkinson

Differentiation of fungal phytopathogens by FT-IR and MALDI-TOF MS

By

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The use of matrix assisted laser desorption ionization time-of-flight mass spectrometry, Fourier transform infrared spectroscopy, and other analytical means of identifying and differentiating microorganisms hold much promise. These analytical tools have been extensively assessed for their ability to differentiate bacteria and fungi. Most of this research has been coordinated in medically relevant microorganisms, but the technology can work just as well with agriculturally important microorganisms. In this thesis, these technologies were reviewed and then subsequently studied for their ability to differentiate *Aspergillus* species (that devastate corn and other crops yearly with aflatoxin contamination), as well as *Macrophomina phaseolina* and *Thielaviopsis basicola* which limit yields on soybean and other crops yearly. With the use of these technologies, harmful plant pathogens could be identified and subsequently treated to improve crop yields and also help to protect our nation and state's food supply.

DEDICATION

To Emily and Logan who have given me all the support to finish my degree and to my parents who got me where I am today, thank you and I love you.

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To Dr. Brown and Dr. Sparks, thank you for giving me the opportunity to complete my research work and helping me find one of the true passions in life. To Dr. Rodriguez thank you for all of your help and advice through the research process. I also want to thank Olga for all of her help in the *Aspergillus* project as well as for her help in teaching me how to conduct myself in a lab. I also want to give one last thank you to Dr. Sparks for always reminding me that "it'll be fine." Finally, thank you all for giving me the opportunity to work at the Mississippi State Chemical Laboratory and getting my professional career started.

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CHAPTER I

A REVIEW OF MASS SPECTROMETRY AND SPECTROSCOPY BASED DIFFERENTIATION OF MICROORGANISMS

Introduction

Traditionally, microorganisms are identified on the basis of genetic sequence, such as polymerase chain reaction (PCR) or real-time polymerase chain reaction (qPCR), biochemically, morphology, and antibody based methods.[1] While, these tests do have their advantages (short-term cost, ease of use, acceptance of methods in literature), there are some fundamental problems associated with using these methods, with a key problem being the skill and experience of the person doing the tests.[2] Though, as with any test, one of the key limiting factors will always be the person performing the tests, which is in part why many tests used for identifying microorganisms are sold as easy to use kits, but are not available for many organisms.

Another key issue in using these standard tests is the time it takes to make a species, or even genus, identification (ID).[3] In an effort to make ID tests high-throughput, much research has been done using matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry and spectroscopy. In the field of spectroscopy, two types of instruments have been extensively studied, including Fourier transform infrared (FT-IR) and Raman spectroscopy. While research has not been limited to these instrument platforms, these have been the most extensively studied. Some other

technologies studied have been laser-induced breakdown spectroscopy and ambient mass spectrometry.[4,5]

While there are multiple mass spectrometry and spectroscopy technologies that have been researched for identifying microorganisms, the most prevalent and relevant to this review are MALDI-TOF MS and FT-IR, while Raman spectroscopy will be briefly highlighted. It is imperative to understand to how the technique is used and how it is currently applied. Moreover, most of these technologies have been extensively utilized and researched for identifying human pathogens, but there is high potential for these technologies to be crossed over into identifying agriculturally relevant microorganisms. Though, the key limiting factor in such technologies is the building and creating libraries with reference spectra unique to each type of instrumentation that are capable of reliable and accurate identifications

MALDI-TOF MS

Matrix assisted laser desorption ionization time-of-flight mass spectrometry has been the most extensively studied and utilized mass spectrometry technology studied for bacterial identification. As such, not a lot of research has been devoted to research to identification of fungi (outside of medically relevant yeast). Thus, a commercial software platform that utilizes MALDI-TOF MS has been developed by Bruker®. This software platform, MALDI BioTyper®, utilizes a proprietary algorithm for distinguishing microorganisms on the basis of mass spectra typically collected from the 2,000 to 20,000 Dalton range. To date, more than 4000 bacteria and fungi species are available for identification using this platform, though bacteria vastly outnumber the amount of fungi

available for identification. Though, to truly an understand the identification, a brief overview of the fundamentals of MALDI are needed.

What is MALDI-TOF MS?

Since 1987, MALDI-TOF MS has been researched for its ability to identify microorganisms.[6] A standard MALDI TOF MS instrument contains a laser, ion source and a TOF mass spectrometer. A sample of interest is placed on a plate and struck with a laser. When the laser "strikes" the sample, energy is transferred, or desorbed, switching the sample from a solid phase to gas phase allowing for the formation of charged molecules to form that go through the TOF mass spectrometer, though for energy transfer to occur for most samples a suitable amount of matrix has to be applied.[7] Examples of different matrices used in MALDI-TOF MS include 2,5-dihydroxybenzoic acid, α-cyano-4-hydroxycinnamic acid, and sinapinic acid. The TOF mass spectrometer differentiates ions on the basis of weight, where larger molecular weight ions pass through the TOF at a slower rate than the lower molecular weight ions as shown in Figure 1.

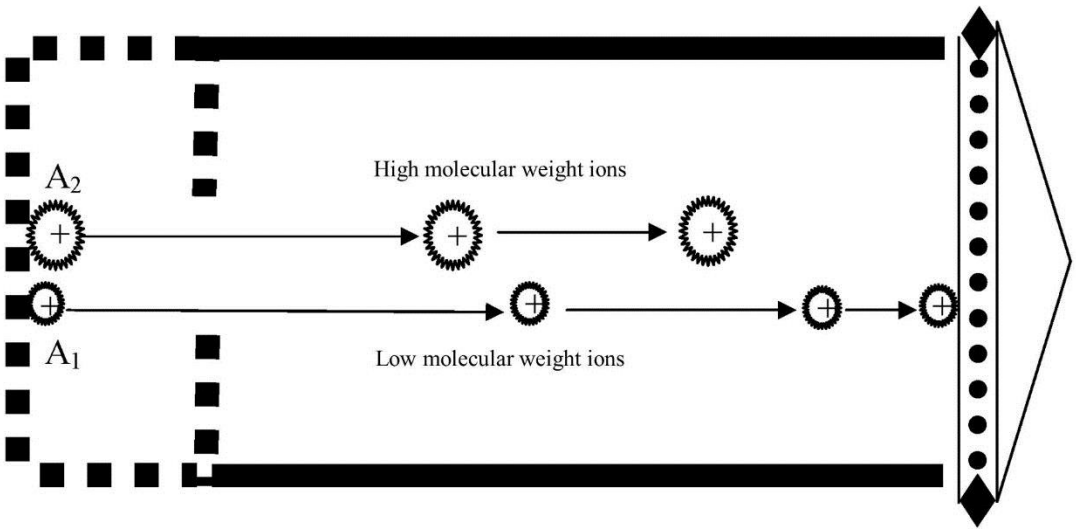

Figure 1 Conventional TOF mass spectrometer

A2 represents a higher molecular weight ion traveling through a TOF mass spectrometer, where A1 represents a lower weight molecular ion passing through the mass spectrometer. Image adapted from Aneed et al. 2009.

Differentiation of Microorganisms

Bacteria

The most widely studied aspect relating to the differentiation of microorganisms and MALDI-TOF MS are the study of bacterial identification. In general, bacterial samples can be prepared in two ways, either whole cell or by acid digestion of the cell wall. The whole cell technique is the simplest requiring only a single colony for to be applied to a sample plate and then covered in matrix.[7] For acid digestion of cell walls, a bacterial sample is placed in water, and rinsed, then centrifuged, supernatant removed, and rinsed with ethanol. After this, the sample is centrifuged again and the supernatant removed, then a weak acid is applied with acetonitrile. It has been noted that the weak acid treatment does have a higher spectral quality as compared to the whole-cell method.[9]

To date, multiple reviews have been published on the topic of using MALDI-TOF MS in identifying bacteria. For a recent and thorough review of findings that correlate with individual genus' and the limitations associated with the technique please see Giebel et al., 2010 or Lay, 2001.[10,11]

Fungi

Much of the research that has been completed with combining MALDI-TOF MS and identification of fungi has involved human pathogenic yeast, such as species belonging to the genus Candida. The sample preparation is much the same as with bacteria, but the pretreatment with ethanol and a weak acid is a necessity.[12]

Overall, studies have focused on Candida species. These studies have yielded that MALDI based identification of yeast is a viable technique for routine clinical laboratories.[3,13-15] Though, research has not been just limited to yeast, MALDI-TOF identification of molds has also been reported. In a study of utilizing over 150 clinical mold samples, an identification rate of 87 % was noted using the acid-digestion technique.[16] In another study looking at the identification of yeast, molds, and dermatophytes (fungi commonly causing skin disease, usually associated as filamentous fungi) it was noted that MALDI based identification was an appropriate tool for identifying the fungi.[17] It was also noted that an decrease in time for identification could be done as well with broth cultures for molds and the dermatophytes.[17]

Overall, MALDI is a practical application with numerous applications for identifying microorganisms in the routine microbiology lab. Most research has revolved around utilizing the technology for medical microbiology, but could have a great impact in agricultural laboratories as well. Thus, as more research and more robust libraries are

built for MALDI, the technology will grow in strength and application for microbiology labs.

Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy is a vibrational spectroscopic technique that utilizes an infrared light source for characterizing chemical compounds. This technology uses the infrared light and passes it through a splitter that effectively splits the light beam into two separate beams. These beams produce a radiation that will cause atoms to vibrate, where individual chemical groups have characteristic vibrations. The data produced from these vibrating atoms within a compound form a unique spectrum for each compound, also called a "fingerprint."[18] It is from this key principle of FT-IR spectroscopy that the basis of identifying and differentiating microorganisms originated. The differences that arise between genus and species of organisms come from the variation present in the cell wall of microorganisms.[19] A typical FT-IR setup is illustrated in Figure 2.

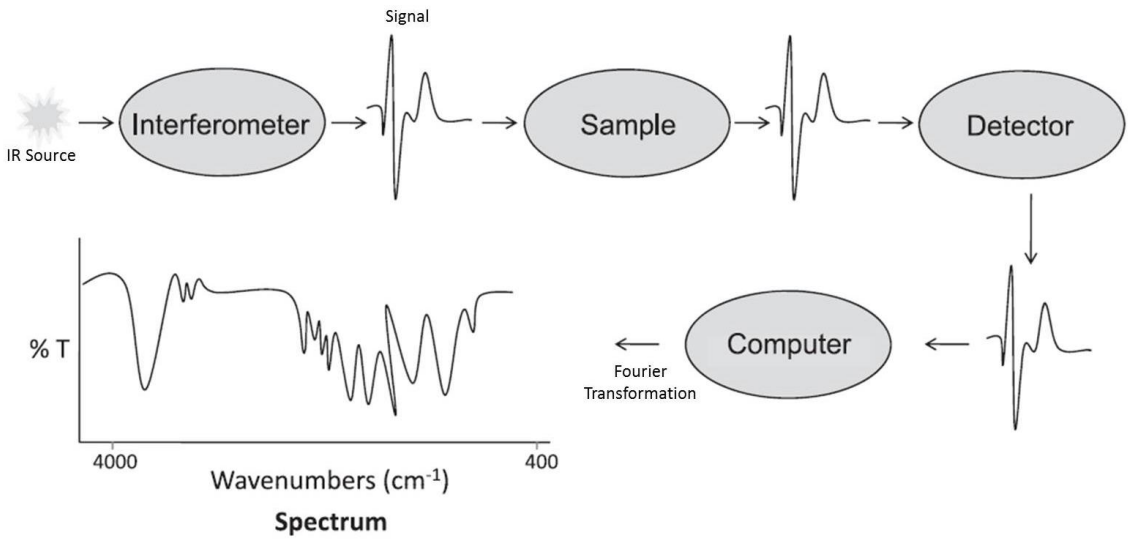

Figure 2 A typical FT-IR setup.

As shown in the diagram, an infrared (IR) source generates the light the passes from the interferometer and generates a signal that will pass through the sample, to the detector, and finally to the computer where Fourier transformation is applied and a FT-IR spectrum is generated. Image adapted from Santos et al., 2010.

The first research involving the use of FT-IR and microbial identification dates back to the 50's.[20] Since the first use of FT-IR for identification, systems have evolved from table-top instruments to now being portable and hand-held.[21] While FT-IR based identification systems enjoy many of the luxuries of MALDI based identification systems, it has one drawback as compared to the mass spectrometry system. The presence of water in great amounts renders spectra collected from FT-IR useless. Though, this can be avoided with a drying step during preparation. There are multiple approaches to analyzing microorganisms by means of FT-IR; it is easier to differentiate on the basis of multi-cell analysis and single cell analysis.

Multi-Cell Analysis

The key difference between multi-cell analysis (macrospectroscopy) and single cell analysis (microspectroscopy) in relation to FT-IR based identification for microorganisms is the presence of a microscope on the instrument, where multi-cell analysis does not contain a microscope. One of the most promising features of multi-cell FT-IR spectroscopy is the ability to use an ATR, attenuated total reflectance, crystal for analyses due to the ability to analyze biofilms directly with little sample preparation.[22] It also allows for analysts to obtain results in real-time and it is non-destructive.[22]

In a study on *Staphylococcus* species, also including *S. aureus*, it was found that FT-IR could differentiate *S. aureus* from other *Staphylococcus* species cultured from raw milk and cheese.[23] In a similar study utilizing pure cultures of *Listeria* species, it was found that *L. monocytogenes* could rapidly differentiated (99.2 % correct identification rate) from other species of *Listeria*.[24] Moving beyond bacteria and into fungi, it was found that *Rhizoctonia*, *Colletotrichum*, *Verticillium*, and *Fusarium* could be rapidly differentiated using FT-IR.[25] In another study utilizing airborne filamentous fungi, it was found that FT-IR was suitable for distinguishing *Aspergillis* and *Penicillium* species.[26] Thus, multi-cell FT-IR spectroscopy does hold promise for the ability to differentiate bacteria and fungi.

Single-Cell Analysis

The distinct advantage of utilizing a single-cell analysis versus using the multi-cell analysis is that it allows for quicker identification through shortened culturing times and smaller sample size.[27] Though, there is increased cost in a microscope coupled FT-IR versus using a "standard" FT-IR. This technique allows for the analysis of single cells, as

the name implies, as well as micro-colonies on plates. On a study of micro-colonies, it was found that micro-spectroscopy could correctly differentiate between gram positive and negative species of bacteria, as well as differentiating species of *Staphylococcus* and other species of yeast with just as little as six hours of culture time.[27] The use of FT-IR micro-spectrometry for differentiating fungi on complex matrices has also been demonstrated on potatoes and in wood.[28,29] Thus, it is apparent that the addition of the microscope to a FT-IR can be more advantageous than compared to one without it.

Raman Spectroscopy

Raman is a technique similar to FT-IR spectroscopy. It also has the key advantage over FT-IR of not being as sensitive to water.[19] It also carries the same ability of using multi-cell and single-cell spectroscopy as well. One key disadvantage of using Raman spectroscopy is that it is inherently weak due to the Raman effect the technique utilizes, though it can be improved through surface-enhanced Raman Spectroscopy (SERS).[30] In a typical SERS experiment, either gold or silver nanoparticles are brought into contact with the microorganisms of interest.[31] These nanoparticles will then enhance (or increase) the absorption of bands present in the spectrum.[31]

In a study of clinical bacteria that had been isolated from bacterial urinary tract infection (UTI) infected patients, it was found that Raman spectroscopy was suitable in differentiating *Escherichia coli*, *Klebsiella species*, *Citrobacter freundii*, *Enterococus* species and *Proteus miabillis*. In a study on macrofungi (mushrooms), Raman spectroscopy was found to be suitable for differentiating spores on the basis of genus (90 % correctly assigned), but not specie.[32] In a single cell study on yeasts, it was found that

Raman was suitable for differentiating clinical yeast species (though, the researchers combined spectra from different cells into averages).

Conclusion

Overall, mass spectrometry and vibrational spectroscopy present a great opportunity for researchers in the field of identifying and differentiating microorganisms. Though, these techniques will not likely replace traditional molecular and phenotype approaches currently in use completely. Yet, they are tools that can aide researchers and diagnosticians alike.

Throughout this review it was also apparent that a bulk of research in these fields has been dominated by medical research. While research has been done in the field of agriculturally relevant microorganisms, it is apparent more needs to be done. These technologies would enable field researchers to utilize the same techniques as those in a medical diagnostic lab. Plus, there would be a key economic incentive in that these techniques could potentially identify phytopathogens (plant pathogens) and would allow an appropriate response to be made (either crop rotation or the addition of fungicides). These technologies, along with others that continue to be discovered and utilized, present a challenge through library building of reference spectrum of microorganisms, but have a key advantage in speed of results and low-cost of sampling. Raman spectroscopy, FT-IR spectroscopy, MALDI-TOF MS , and other technologies present a great utility in the identification of multiple microorganisms, including bacteria, yeast, filamentous fungi, and macro-fungi.

CHAPTER II

DIFFERENTIATION OF AFLATOXIGENIC AND NON-AFLATOXIGENIC STRAINS OF ASPERGILLI BY FT-IR SPECTROSCOPY

Introduction

According to the World Health Organization (WHO) approximately 25% of the world's food supply is contaminated with mycotoxins. In the USA, crop damages due to mycotoxin contamination translate to 0.5 to 5 billion dollars losses per year.[34] Examples of mycotoxins include aflatoxins, fumonisins, trichothecenes, ochratoxin, etc. Aflatoxins (B1, B2, G1 and G2) are produced as secondary metabolites by certain strains of filamentous fungi, such as *A. flavus* and *A. parasiticus*, and are the major contributors to the contamination of food and animal feed. Aflatoxins are very toxic and have carcinogenic, teratogenic, and oestrogenic effects. In fact, aflatoxin has been classified as a Group I carcinogen.[35] Amounts of aflatoxins in foods and animal feeds are strictly regulated throughout most of the world. In the USA, FDA-approved aflatoxin levels were set below 20$\mu g \cdot kg^{-1}$.

Aspergillus flavus and *A. paraciticus* infect several agricultural commodities, such as maize and peanuts. Diverse communities of these fungi can reside together in the same environment. Both fungal species can be divided into two major groups of strains based on morphological, genetic, and physiological criteria. One group of strains is a producer of high levels of aflatoxins; while the other produces little or no aflatoxins. To control

aflatoxin contamination of crops, non-toxigenic *A. flavus* isolates are often employed to competitively inhibit aflatoxigenic Aspergillus spp., thus limiting aflatoxin contamination.[36,37] Under the commercial name Afla-guard®, non-toxigenic *A. flavus* strains (developed from NRRL 21882), prevent an establishment of toxigenic Aspergillus strains in the field.[36] Implementation of such a preventive approach, however, requires a rapid and cost effective technique that could reliably identify and differentiate between the toxigenic and non-toxigenic *Aspergillus* strains.

FT-IR spectroscopy is a methodology capable of identifying microorganisms based on their spectral profiles. These profiles are obtained from the interactions of various chemical groups present on the surfaces of the cells with mid-infrared light.[19] Fourier transform infrared spectroscopy does not require highly specialized skills and it yields reproducible results. It has already been shown to differentiate Aspergilli at the specie and strain levels; however, no differentiation between toxigenic and non-toxigenic strains was yet documented.[38] In this study, both aflatoxin-producing and non-producing strains of *A. parasiticus* and *A. flavus* were successfully differentiated using FT-IR and standard statistical multivariate methods. Such differentiation could prove valuable for monitoring the crops for aflatoxigenic and non-aflatoxigenic Aspergilllus strains, especially those treated with a competitive, non-toxigenic strain to ensure that crop safety is being maintained.

Experimental

Materials

For use in this study, HPLC grade methanol (Fisher Chemical; Hampton, NH) was used and all water used was sterile, double distilled.

Aspergillus strains and cultivation

Aflatoxigenic strains of *Aspergillus flavus* ATCC 26768, ATCC 26769, ATCC 26770, ATCC 26771, and ATCC 34689 and *Aspergillus parasiticus* ATCC 26691, ATCC 28285, ATCC 26690, ATCC 26692 and ATCC 26862 were obtained from American Type Culture Collection (ATCC; Manassas, VA). Atoxigenic *A. flavus* strains NRRL 21882, NRRL 29506, and NPL NC5.2 were provided by Dr. Gary Windham (USDA, ARS, Corn Host Plant Resistance Unit, Mississippi State, MS). Strains NRRL 21368 and NRRL 35743 (NRRL 2947A-20-Control) were obtained from Dr. Bruce Horn (National Peanut Research Laboratory, USDA, ARS, Dawson, GA). The atoxigenic strains of *A. parasiticus* ATCC 56857, ATCC 56858, ATCC 56859, and ATCC 56860 were also obtained from ATCC, and *A. parasiticus* NRRL 21369 was obtained from Dr. Bruce Horn. All fungi were grown from 30% glycerol cell stocks. For FT-IR analysis, strains were revived on potato dextrose agar (PDA) (Remel; Lenexa, KS) for 6 days at 25 ˚C. After revival, they were plated on 4 separate PDA plates (biological replicates) and grown under the same conditions. Each strain produced 12 samples for the study with the exception of *A. flavus* NRRL 29506 (10), *A. flavus* NPL NC5.2 (9), *A. parasiticus* ATCC 26862 (11), and *A. parasiticus* ATCC 26690 (10). This resulted in the use of 196 samples for the presented study.

Sample Preparation

From each biological replicate plate, three samples (technical replicates) were prepared as follows: 6 mL of sterile double distilled (dd) H2O were deposited directly onto the agar plates and fungal spores were harvested by gentle scratching using a cell lifter. One milliliter of the cell-containing solution was transferred to 3 separate 1.5 mL

centrifuge tubes and centrifuged at 21,000xg for two minutes. The supernatant was discarded and the cells were washed with 1 mL of sterile ddH2O. This process was repeated 2 more times and was followed by 3 washes with 1 mL of 100 % methanol. Vigorous vortexing was used between the washes. After the final wash, 1 mL of 100 % methanol was added, samples were vortexed, and subjected to ten-minute sonication using a Branson 1510 sonicator (Branson Ultrasonics Corp., Danbury, CT).

FT-IR Spectroscopy

FT-IR spectroscopy was performed using a nitrogen gas flushed NicoletTM 6700 FT-IR spectrometer (Thermo Scientific, San Jose, CA) equipped with a liquid nitrogen cooled MCT high D* detector, a KBr beam splitter, and the Smart ARK^{TM} (Thermo Scientific) accessory. For each FT-IR analysis, 200 µL of the sonicated cell solution were deposited onto a ZnSe Horizontal Attenuated Total Reflectance (HATR) crystal with an angle of incidence of 45˚ that allows for 10 reflections of infrared light to pass through the crystal for each scan. A total of thirty-two scans per spectra, measuring absorbance from 4000 cm-1 to 500 cm-1 were acquired and averaged. Background spectra were collected before each analysis and they were subtracted from each sample. The spectra region between 1800 cm-1 and 800 cm-1 was selected for all figures and statistical analysis. A typical spectrum collected for use in this study is shown in Figure 3.

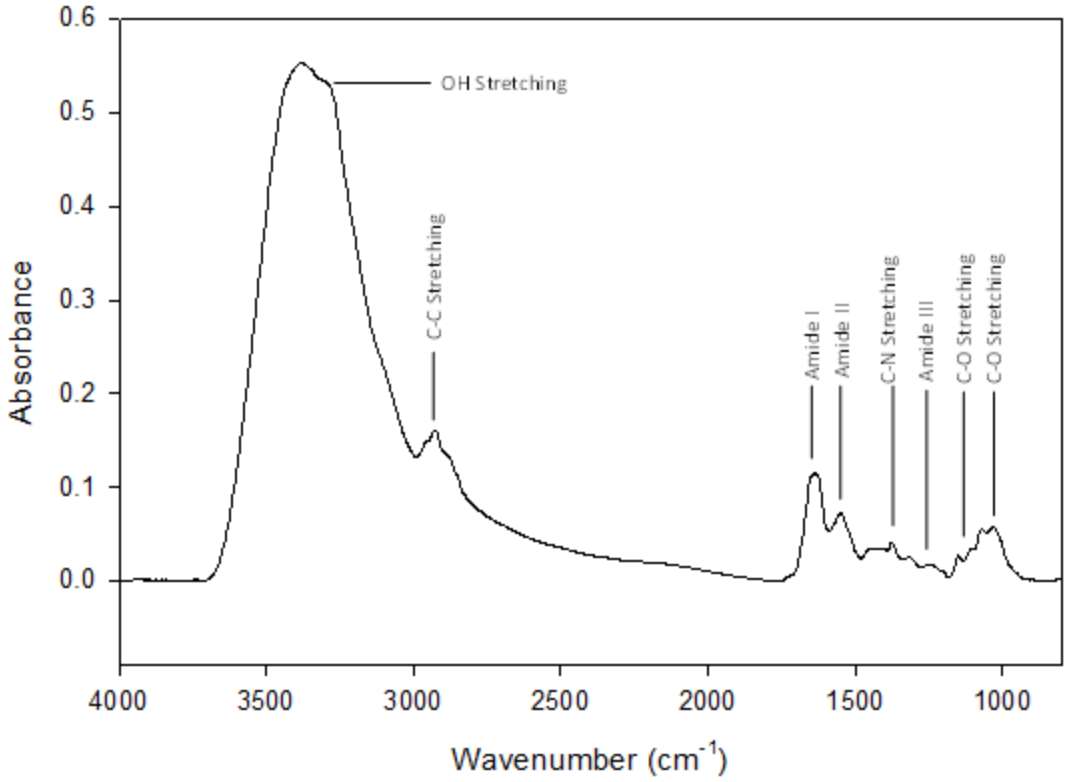

Figure 3 A Typical Spectrum Collected for Study

Spectrum was collected from 4000 cm^{-1} to 800 cm^{-1} and has been baseline corrected.

Multivariate Statistics

Spectra were baseline corrected and further corrected for 10 bounces using the advanced ATR correction function in the Thermo Scientific FT-IR Software, OMNIC. Spectra were then exported to The Unscrambler X v10.2 software (Camo Software, Oslo, Norway). The spectra were normalized to the area under the curve and spectra were then converted to the 1st derivative using the Sovitzky-Golay algorithm at 15 points. After data conversion, the spectra were mean-square centered and subjected to principal component analysis (PCA) and cluster analysis by Ward's method.

Results and Discussion

The rapid and accurate identification of *Aspergillus* strains is of considerable importance in agriculture, food and animal feed production as well as clinical medicine. It is usually performed by tedious subjective investigations of macro- and microscopic examinations of their morphology, culture, and spore characteristics and using traditional methods such as biochemical assays and polymerase chain reaction.[26,27,39] Recently, FT-IR spectroscopy has been extensively used to identify microorganisms based on their spectral profiles, which are obtained from interaction of various chemical groups present on the surfaces of the cells with infrared light.[19]

In the presented study, implementing FT-IR spectroscopy, 20 Aspergilli strains were fingerprinted and differentiated based on their toxigenicity. Using the 1st derivative spectra, four distinct clusters, with some overlap between toxigenicity and specie, were produced employing the 3-D PCA plot of PC-1, PC-2, and PC-3 (Figure 4). The usage of first derivative spectra rather than the absorbance spectra reduces the amount of baseline variation present between the spectra, significantly enhancing the reproducibility of results. The principle components used for visualizing the data accounted for >99% of the observed variance in the spectra. Comparison of the individual species based on toxigenicity yielded similar results as shown in Figures 5 and 6. *Aspergillus flavus* strains are readily differentiated as shown in figure 4 on the basis of toxigenicity, yet the same cannot be said for *A. parasiticus* strains. There is a large overlap between toxigenic and non-toxigenic strains. This is likely due to the little variation present between the individual strains. Though, there is a noticeable trend in the analysis, where a majority of the toxigenic strains plotted above 0 on the Y-axis (PC-2), and almost all the non-

toxigenic strains plotted below 0 on the Y-axis. This correlates with the data obtained in Figure 6, where the same trend also occurred, where all toxigenic strains grouped above 0 on the Y-axis and all but one sample in the non-toxigenic strains plotted below 0 on the 2nd principal component for *A. flavus* strains. Thus, it appears that there is correlation with PC-2 and aflatoxigenicity.

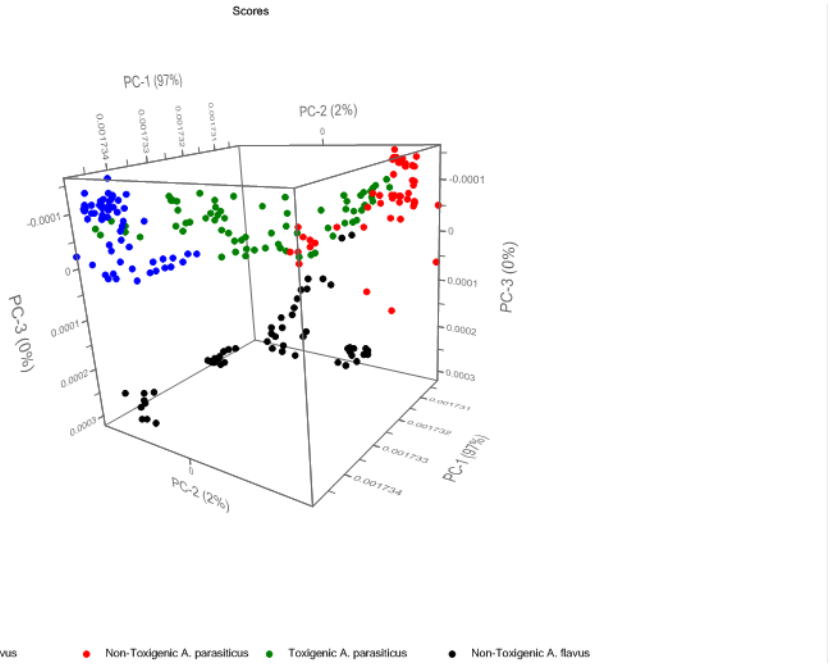

Figure 4 PCA analysis of all *A. parasitcus* and *A. flavus* samples

The 3D plot utilizes PC's 1, 2, and 3 that accounts for >99% of the variance observed. Depicted here non-toxigenic *A. flavus* (black), toxigenic *A. flavus* (blue), non-toxigenic *A. parasiticus* (red), and toxigenic *A. parasiticus* (green).

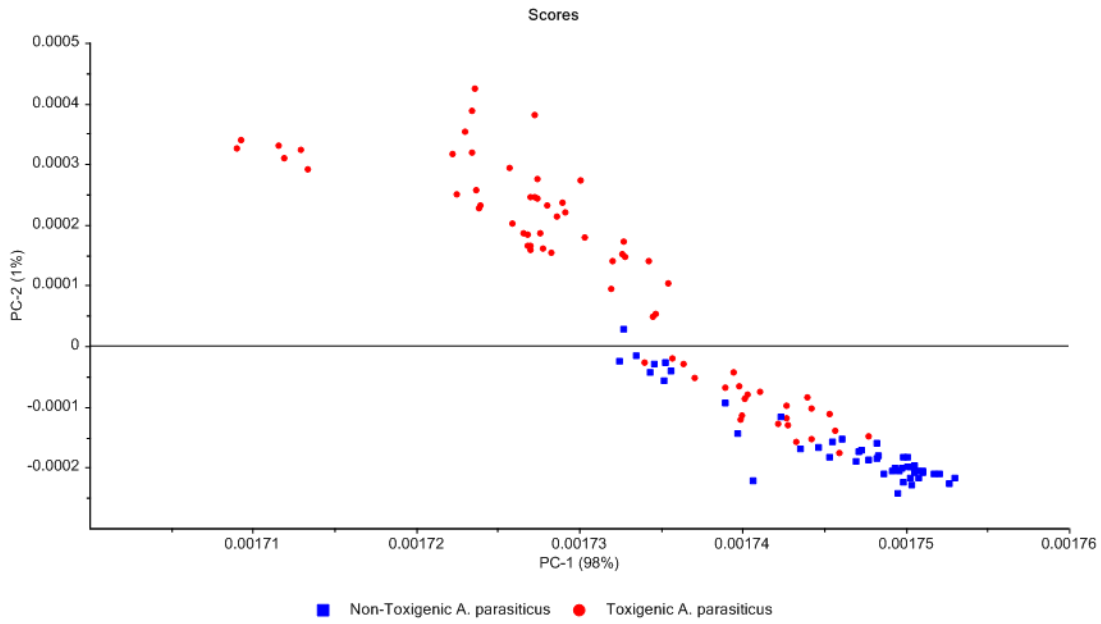

Figure 5 PCA of *A. parasiticus* strains

The 2D plot utilizes PC's 1 and 2, that account for >99% of the observed variance. Labeled in blue is the non-toxigenic *A. parasiticus* and red is toxigenic *A. parasiticus* strains.

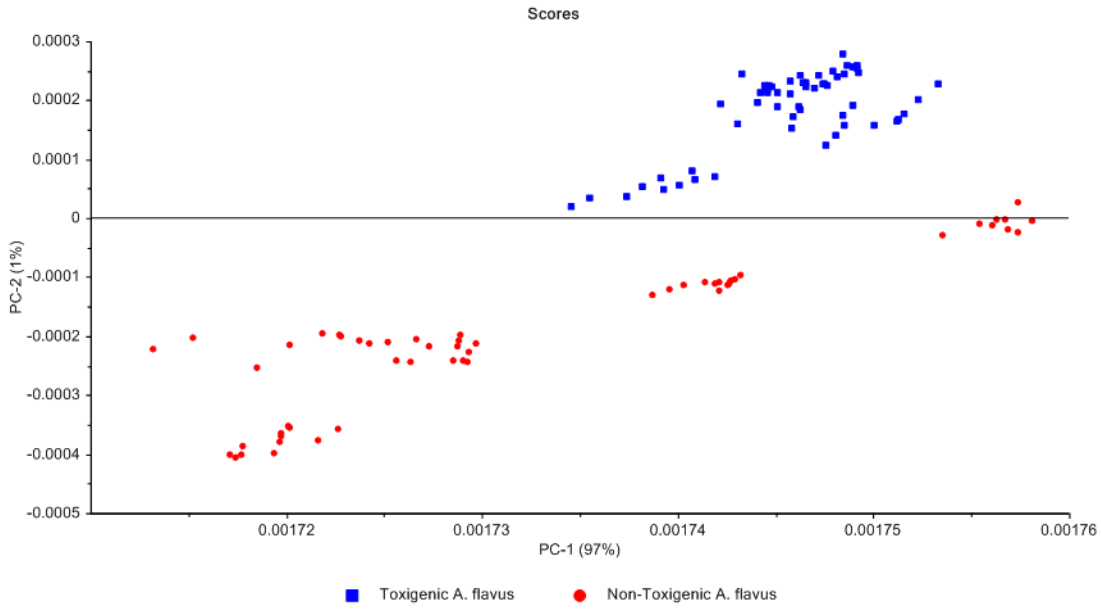

Figure 6 PCA of *A. flavus* strains

The 2D plot utilizes the PC's 1 and 2 that account for >99% of the observed variance. Labeled in red are the non-toxigenic *A. flavus* strains and labeled in blue are the toxigenic *A. flavus* strains.

After PCA, cluster analysis was performed using Ward's algorithm. It allowed for the measurement of differences between multiple spectra in a metric manner. This analysis emphasizes the differences between spectra that are not readily observed. Cluster analysis is summarized in Tables I and II, where the differentiation of the two species is possible on the basis of mid-IR spectra for specie and toxigenicity differentiation, producing 4 distinct clusters. Cluster 1 contains all 5 of the non-toxigenic strains of *A. parasiticus*, along with all 12 samples of the toxigenic strain of *A. parasiticus* (ATCC 26992) plus two samples of non-toxigenic *A. flavus* NRRL 29506, which the latter two clustered away from the non-toxigenic *A. parasitcus* strains. The second cluster contained 4 toxigenic strains of *A. flavus* (ATCC 26768, ATCC 26770, ATCC 26771, and ATCC 34689) along with 6 samples of toxigenic *A. paraisiticus* ATCC 26692. The third cluster contained all 10 samples of the toxigenic *A. flavus* ATCC 26769 along with all the samples belonging to the toxigenic *A. parsiticus* strains ATCC 26690, ATCC 26862, and ATCC 28285 along with the other 6 samples of *A. parasiticus* ATCC 26691. The 4th cluster contained all the non-toxigenic strains of *A. flavus* (NRRL 29506, ATCC 35743, NPL NC5.2, ATCC 21368, and NRRL 21882). Thus, differentiation on the basis of toxigenicity was completed with all but one strain of *A. parasiticus* (ATCC 26692). This lone exception is likely due to the strong similarity in cell wall present between this strain and the other non-toxigenic strains of *A. parasiticus*. It is important to note that there was a strong enough difference in the samples that the 12 samples did cluster away from the non-toxigenic strains, but did cluster along with 2 samples of a non-toxigenic *A. flavus* strain. Though, Wei & Jong (1986) found in a study of aflatoxin production across three matrices (rice, peanuts, and yeast extract with supplements medium), that *A. parasiticus*

26692 is a minimal producer of aflatoxin, where only aflatoxin B1 was found to be produced in peanuts.[44] In our research we did not attempt to quantify aflatoxin production of the strains used, but it is likely that the strain either did not produce aflatoxin or was produced in extremely low amounts.

Table 1 Cluster analysis of Aspergilli strains.

	Number of Samples per Cluster			
Specie & Strain	1	2	3	4
AF 29506	2	0	0	8
AF 35743	0	0	0	12
AF NPL NC5.2	0	0	0	9
AF 21368	0	0	0	12
AF 21882	0	0	0	12
AF 26768*	0	12	0	0
AF 26769*	0	0	10	0
AF 26770*	0	12	0	0
AF 26771*	0	12	0	0
AF 34689*	0	12	0	0
AP 21369	12	0	0	0
AP 56857	12	0	0	0
AP 56858	12	0	0	0
AP 56859	12	0	0	0
AP 56860	12	0	0	0
AP 26692*	12	0	0	0
AP 26690*	0	0	10	0
AP 26691*	0	6	6	0
AP 26862*	0	0	11	0
AP 28285*	0	0	12	0
Total	74	54	49	53

Note: Four clusters were produced from the cluster analysis. The number of samples per cluster is indicated by specie and strain. A "*" indicates a toxigenic strain of *Aspergillus* and AP designates *Aspergillus parasiticus* and AF designates *Aspergillus flavus*.

Conclusion

The reliable differentiation between aflatoxigenic and non-aflatoxigenic *Aspergillus* spp. by FT-IR is one of the most critical parameters that could be exploited by the agricultural industry. This technique could prove vital for crops treated with Afla-guard® as well as other food producing industries that could suffer tremendous losses due to aflatoxin contamination by toxigenic strains of *Aspergillus*. We show that FT-IR can successfully differentiate the two Aspergilli species on the basis of toxigencity and specie, and our findings also correlate with previously published studies, where similar results were obtained for other microorganism such as bacteria, yeast, and filamentous fungi.[19,23-29,38-43] Overall, analysis of this nature could be used for a rapid screening of *Aspergillus*-contaminated products.

Table 2 Clustering of specie and toxigenicity by cluster

	Number of Samples in Cluster Based on Specie and Toxigenicity				
Cluster	*A. parasiticus*	*A. flavus*	Toxigenic	Non-Toxigenic	Total
1	2	72	12	62	74
2	48	6	54	0	54
3	39	10	49	0	49
4	0	53	0	53	53

In addition to strain differentiation, the sample preparation procedure was slightly improved by omitting water in the final washing solution and using 100 % methanol instead. Water content in samples significantly extends the time of air-drying and often requires the usage of a vacuum dryer or an oven to completely dry the sample, prolonging the time of analysis.[23,25,40,41] It also can result in elevated background response, which can

mask the spectra of interest. Moreover, the high surface density of water (72.8 dyn/cm at 20˚C) makes it difficult to use due to beading that can decrease the homogeneity of a sample, increasing the drying time. In the presented study, it was shown that 100 % methanol can serve as a suitable solvent for multi-cellular spectroscopy. Due to its several advantages (as opposed to water) such as quick air-drying time without the need of vacuum/oven, lower surface tension, etc., methanol-based sample preparation resulted in highly reproducible FT-IR spectra.

Contamination of maize (*Zea mays*) and other crops with aflatoxins is a persistent problem that can pose serious health hazards to both humans and animals. To reduce this contamination, non-aflatoxigenic strains of *Aspergillus* are being used to competitively inhibit aflatoxin-producing strains. To monitor these strains, a FT-IR based technique has been developed that allows for the differentiation of *Aspergillus* strains on the basis of aflatoxigenicity. This will allow for rapid detection of potential health risks, while also ensuring competitive inhibition by biological control agents such as Afla-guard® is occurring in treated fields.

CHAPTER III

THE USE OF MALDI-TOF MS, FT-IR, AND LIBS FOR THE DIFFERENTIATION OF TWO FUNGAL SOYBEAN PATHOGENS

Introduction

In 2011, soybeans (*Glycine max*) accounted for over $800 million worth of the agricultural commodities in the state of Mississippi.[45] Yet, throughout the nation, soybean yields have been decreased by phytopathogens.[46] In particular, *Marcophomina phaseolina* and *Thielaviopsis basicola* cause significant damage to soybean crops around the southeast. These two phytopathogens cause charcoal rot and black root rot, respectively, to soybean plants and decreasing yields while also decreasing seed quality.

Currently, *M. phaseolina* is identified by morphology, but there has been a PCR protocol developed to identify the pathogen.[47] With respect to *T. basicola*, it is detected the same *M. phaseolina* with morphology, but a real-time PCR assay has also been developed for detection.[48] Yet, both identifying by morphology and PCR do have their drawbacks, such as the years of experience to identify the pathogens by microscope or the inherent costs of PCR.

In the last 20 years, advances have been made in the field of analytical chemistry that has shown to be successful in the identification of microorganisms. Of these technologies, matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), Fourier transform infrared spectroscopy, and laser induced

breakdown spectroscopy (LIBS) have shown promise in their ability to identify and differentiate microorganisms.[4,12,19] Each of these techniques are cheap on a per sample basis and have the capability to be high-throughput. In the presented study, MALDI-TOF MS, FT-IR, and LIBS were used to characterize and differentiate *M. phaseolina* and *T. basicola* on the basis spectral profiles produced from each analytical technique.

Experimental

Fungi Growth and Cultivation

Both, *M. phaseolina* and *T. basicola* were obtained from Gabe Scumbiato from the Mississippi State University Extension Service located in Stoneville, MS. For all experiments done, both species were cultured on potato dextrose agar (PDA) (Remel; Lenexa, KS) for five days at 25 ˚C. For MALDI-TOF MS, plates were sub-cultured into potato dextrose broth and incubated at 25 ˚C on a rotator for two days, then used for analysis. For LIBS and FT-IR, the primary cultures were used for direct analysis.

MALDI-TOF MS

Sample preparation

Sub-cultures of both species were removed from the rotator and allowed to rest for 10 minutes. Then, excess broth was removed and 1 mL of the fungi and broth mixture were placed in 1.5 mL centrifuge tubes. A total of 4 sub-cultures of each were used and placed in individual tubes. Samples were spun for two minutes at 21,000 RCF. The supernatant was removed and 1 mL of HPLC grade water was added and vortexed for 30 seconds. This step was then repeated. After disposal of supernatant, 300 µL of HPLC grade water was added and cells were re-suspended, then 800 µL of 100 % ethanol was

added and the sample vortexed for 30 seconds. This step was then repeated. After the final portion of ethanol was added, the supernatant was removed and the pellet was allowed to dry in a chemical hood for 30 minutes, or until dry, at room temperature. After drying, 100 µL of 80 % optima LC/MS formic acid (Fisher Chemical, Hampton, NJ) added and the cells vortexed for 30 seconds or until the pellet was resuspended. Then 100 uL of HPLC grade acetonitrile was added and vortexed for 30 seconds. Samples were then centrifuged for 2 minutes at 21,000 RCF. One microliter of the sample was then spotted on a 96-well polished steel plate in 8 different wells and allowed to dry. Finally, after drying samples were covered with 1 µL of α-Cyano-4-hydroxycinnamic acid (CHCA).

Instrumentation

Analysis for both species was performed with a Bruker Microflex MALDI-TOF MS in linear, positive mode. The software used for spectrum acquisition was FlexControl (Bruker Daltonics, Billerica, MA), v. 3.3. Spectra were collected between 2 kDa and 20 kDa and 240 spectra were collected to form an individual spectrum. The instrument was calibrated using the IVD bacteria test standard (Bruker Daltonics), which was prepared according to manufacturer's instruction. After spectrum collection, the data was exported to BioTyper (Bruker Daltonics) for multivariate analysis.

Statistics

In BioTyper, samples were normalized by maximum normalization, then smoothed by Gaussian filter, and subjected to cluster analysis by Ward's method.

FT-IR

Sample preparation

Using the primary culture, samples were scrapped from the agarose plate using a cell lifter and 5 mL of double distilled water. One milliliter of the cell containing water was the placed in a 1.5 mL centrifuge tube, and three samples were collected from each plate. Samples were then centrifuged at 21,000 RCF for two minutes and the supernatant was drained and 1 mL of double distilled water was added, then vortexed for 30 seconds, and centrifuged again at the same RCF and time. This step was then repeated. After the final water washing step the supernatant was removed and 1 mL of 100 % methanol (Fischer Chemical) was added. The sample was vortexed and centrifuged at the same RCF and time. This step was then repeated. After the final washing, the methanol was removed and 1 mL of 100% methanol was added again prior to analysis.

Instrumentation

FT-IR spectroscopy was performed using a nitrogen gas flushed Nicolet^{TM} 6700 FT-IR spectrometer (Thermo Scientific, San Jose, CA) equipped with a liquid nitrogen cooled MCT high D* detector, a KBr beam splitter, and the Smart ARK^{TM} (Thermo Scientific) accessory. For each FT-IR analysis, 200 µL of the sonicated cell solution were deposited onto a ZnSe Horizontal Attenuated Total Reflectance (HATR) crystal with an angle of incidence of 45˚ that allows for 10 reflections of infrared light to pass through the crystal for each scan. A total of thirty-two scans per spectra, measuring absorbance from 4000 cm^{-1} to 500 cm^{-1} were acquired and averaged. Background spectra were collected before each analysis and they were subtracted from each sample. The spectra

region between 1800 cm^{-1} and 800 cm^{-1} was selected for all figures and statistical analysis.

Statistics

Spectra were baseline corrected and further corrected for 10 bounces using the advanced ATR correction function in the Thermo Scientific FT-IR Software, OMNIC. Spectra were then exported to The Unscrambler X v10.2 software (Camo Software, Oslo, Norway). The spectra were normalized to the area under the curve and spectra were then converted to the 1st derivative using the Sovitzky-Golay algorithm at 15 points. After data conversion, the spectra were mean-square centered and subjected to principal component analysis (PCA).

LIBS

Sample preparation

Samples were prepped by water and methanol washing just as in FT-IR analysis. After samples were prepped, they were stored at 5 ˚C until ready for shipment. Samples were shipped to Applied Spectra (Fremont, CA) for LIBS analysis. Samples were then spotted on Whatman 7.0 cm filter paper and left to dry for 4 days in a fume hood.

Instrumentation

All LIBS analyses were performed by Applied Spectra using a RT-100 EC (UV) LIBS. Two samples of each species were analyzed from 25 locations on the filter paper in a 5 by 5 grid along with a section of filter paper with no sample present to serve as a control.

Results and Discussion

MALDI-TOF MS

As shown in Figures 7 and 8, *T. basicola* and *M. phaseolina* produced similar mass spectra in the measured range and most of the signals accumulated in the mass spectra between the area from 2000 Da and 8000 Da. There was a lot of noise present in spectrum in the region from 2000 Da and ~5000 Da in the *M. phaseolina* samples.

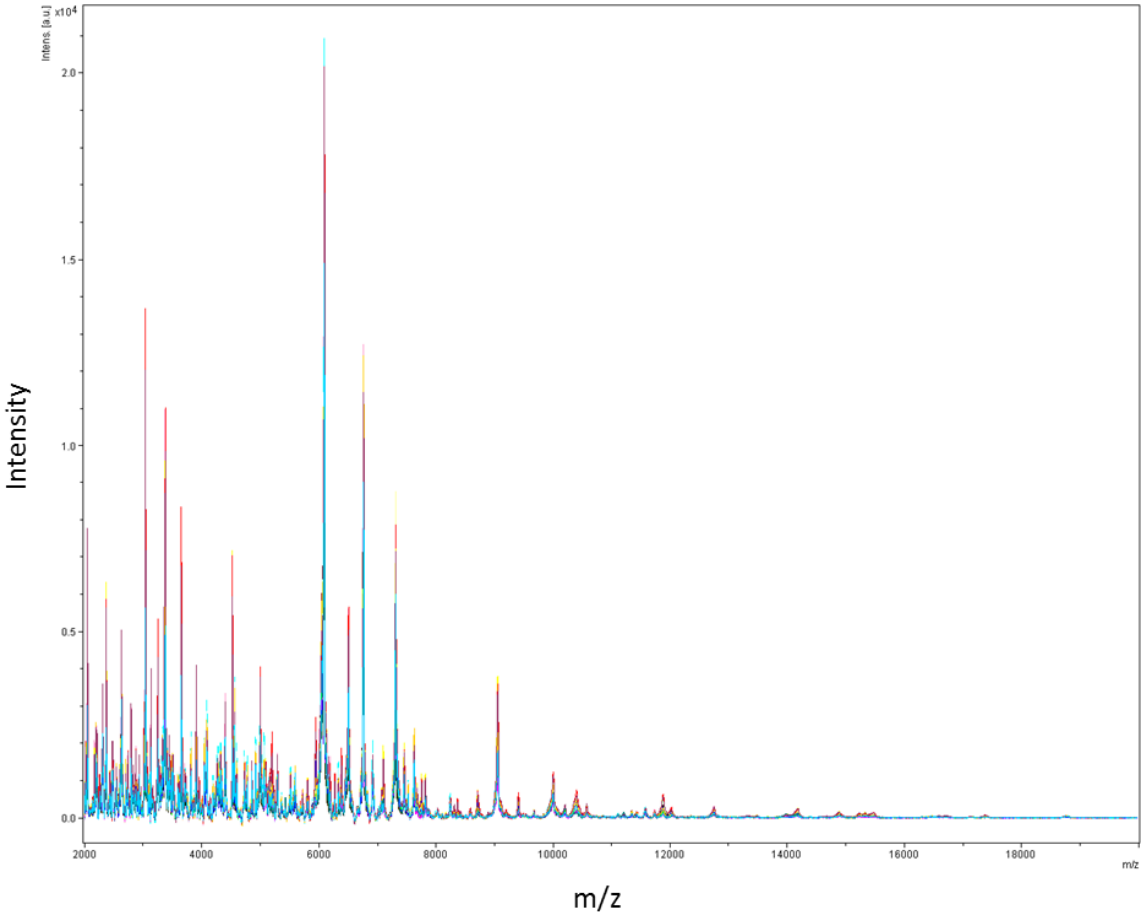

Figure 7 *Thelaviopsis basicola* MALDI-TOF MS spectrum

This figure represents 24 individual spectrums collected from 3 different samples of *T. basicola*. The spectra were baseline corrected and smoothed.

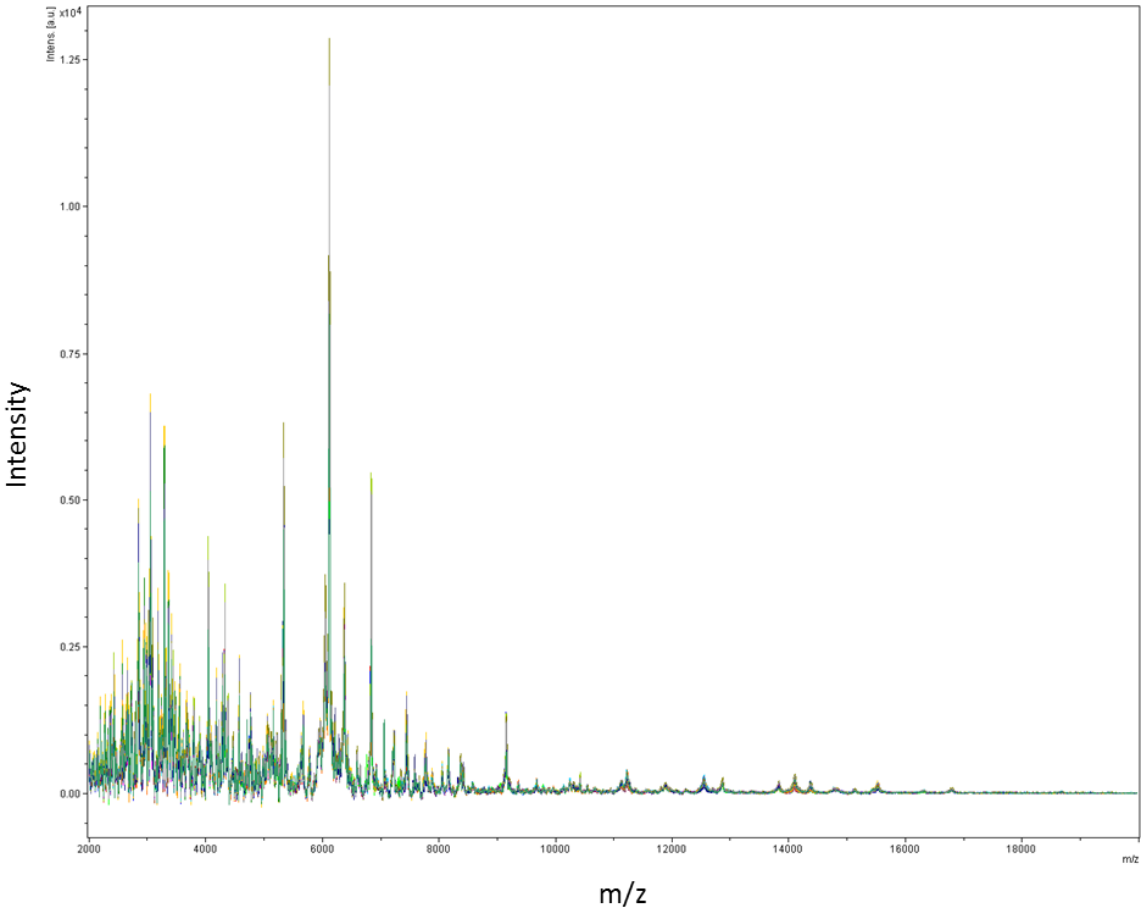

Figure 8 *Macrophomina phaseolina* MALDI-TOF MS spectrum

This figure represents 24 individual spectrums collect from 3 different samples of *M. phaseolina* 24 different wells. The spectra were baseline corrected and smoothed.

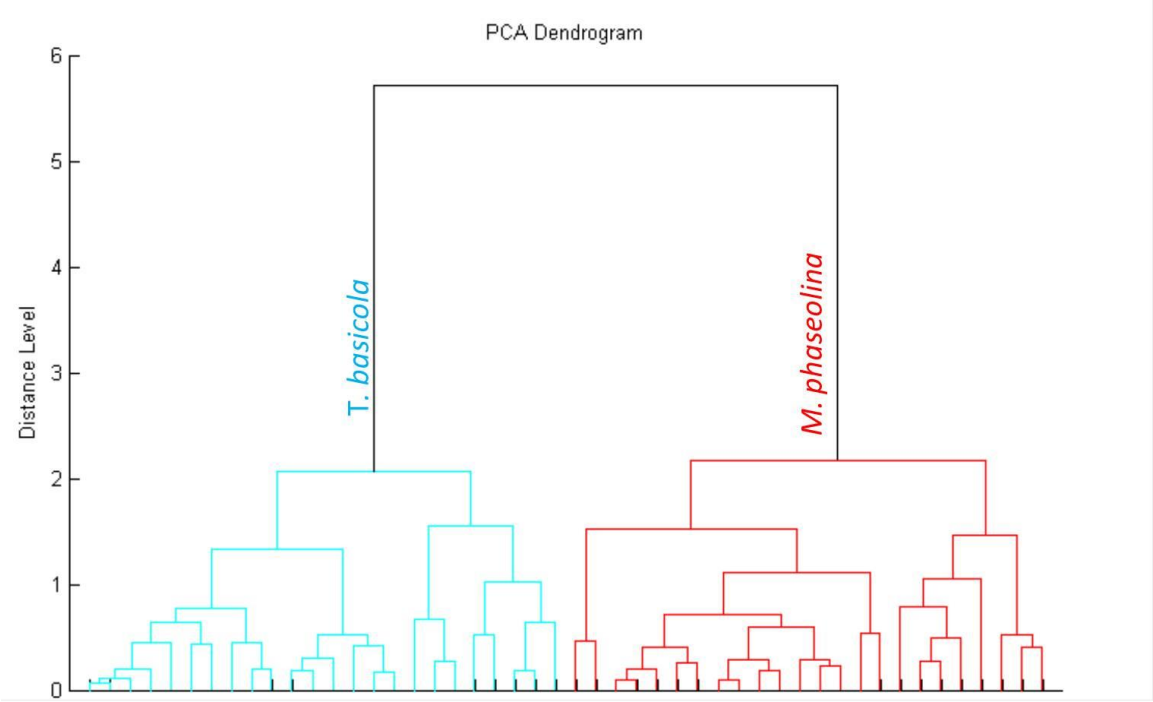

Figure 9 Differentiation of *M. phaseolina* and *T. basicola* by cluster analysis. Three samples, 8 different spectra from individual spots, of both *M. phaseolina* (red) and *T. basicola* (blue) were used for cluster analysis.

From the cluster analysis shown in Figure 9 it was apparent that there was clear differentiation between the two species. There was a ~6 distance levels between the two species. Looking at the individual species, three mini clusters were formed in each main cluster of species. This is likely due to the sample to sample variation, but it does not impact the differentiation of the two species of filamentous fungi. Overall, MALDI-TOF MS seems suitable for differentiating the two species, but there is sample to sample variation present.

FT-IR Spectroscopy

The analysis of *M. phaseolina* and *T. basicola* by FT-IR spectroscopy was straightforward. As shown in Figures 10 and 11 the fungi produced different spectra. The

most notable difference between the two species' spectra is the presence of the intense and sharp band at ~1750 cm^{-1} in *M. phaseolina* samples, but not in the *T. basicola* samples. Though, this sharp band was not present in 3 samples of *M. phaseolina*.

The two species' FT-IR spectra were also subjected to PCA. From figure 12 it is apparent there is a clear differentiation between the two species where all 12 samples of *T. basicola* grouped above on the 2nd principle component and all samples of *M. phaseolina* grouped below 0 on the second principle component, with the exception of 3 samples of *M. phaseolina* that grouped closer to the *T. basicola* samples above 0 on the 2nd principle component. These 3 samples are the same three that did not produce the sharp band around 1750 cm^{-1}. It is likely this caused of the variation present between these 3 samples and the other 9. This variation was likely produced from researcher error or instrument error.

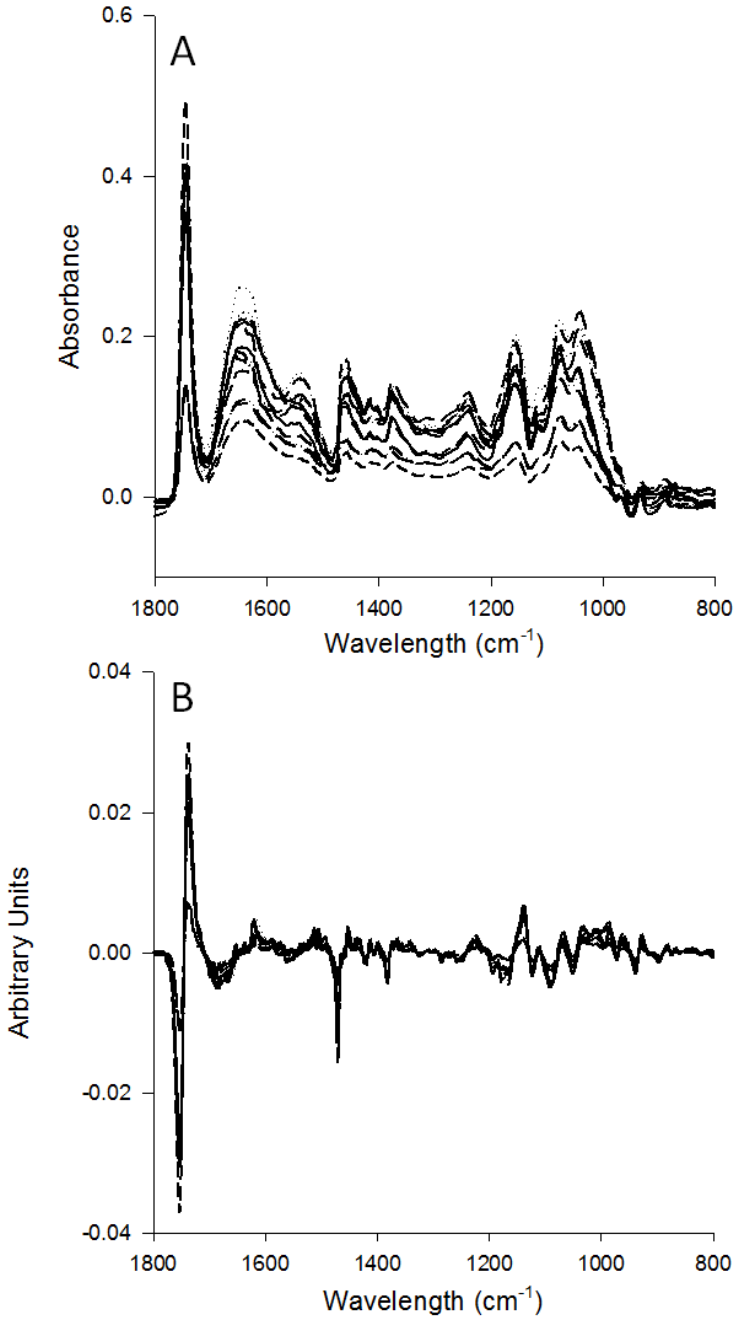

Figure 10 *Macrophomina phaseolina* FT-IR spectra.

"A" shows the raw spectrum and "B" shows the 1st derivative spectrum. 12 spectrum are shown in both "A" and "B."

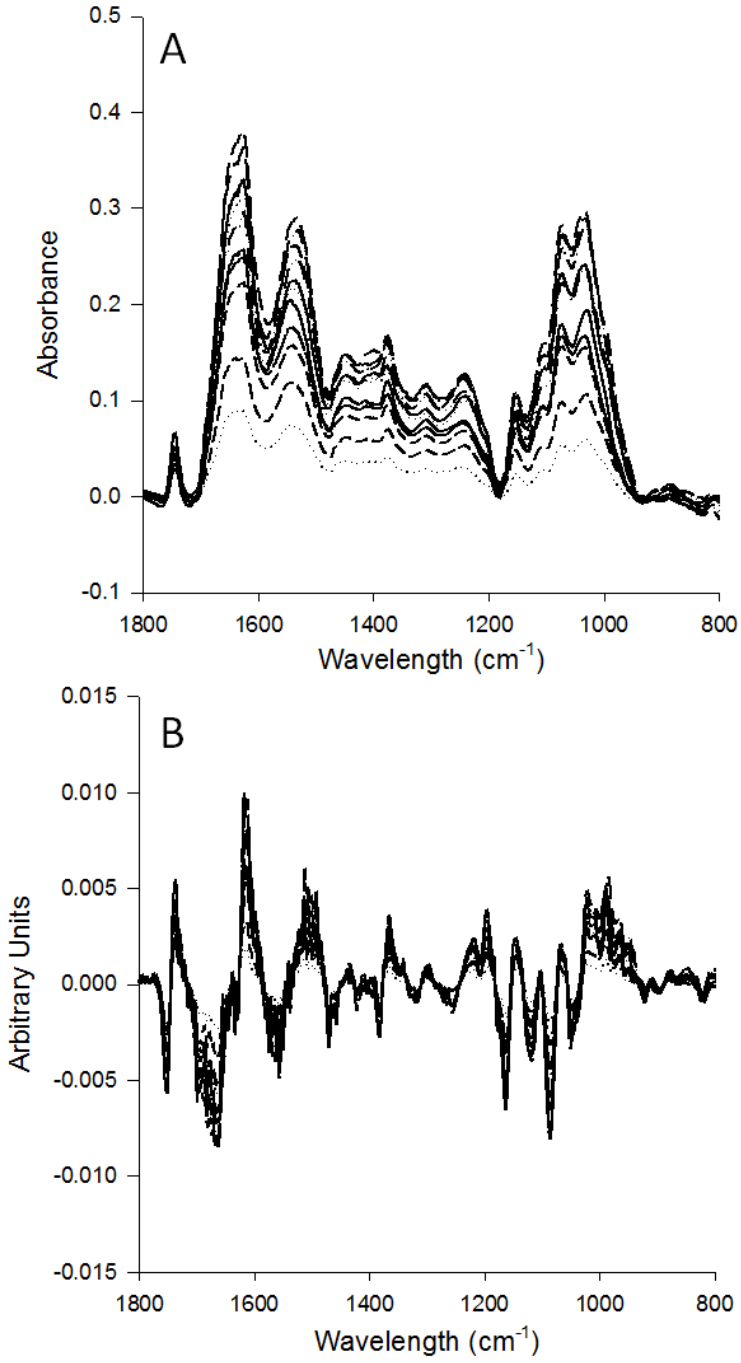

Figure 11 *Thielaviopsis basicola* FT-IR spectra.

"A" illustrates raw spectra, where "B" shows 1st derivative spectra. Twelve samples are shown in both "A" and "B."

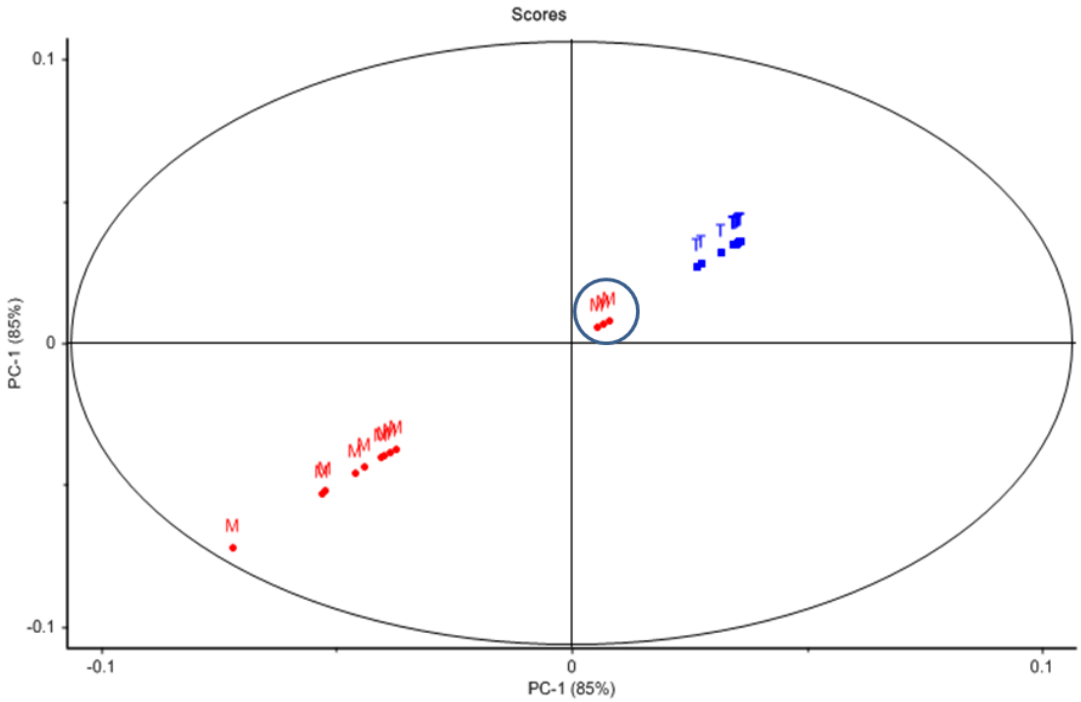

Figure 12 PCA of *M. phaseolina* and *T. basicola* FT-IR spectra.

Macrophomina phaseolina samples are shown in red, while *T.basicola* samples are shown in blue. The circle highlights three M. phaseonlina samples that did not produce a sharp band around 1750 cm^{-1}.

LIBS

The LIBS spectra for both samples illustrated slight differences in the amount of elements present in and on the cells. As shown in Figure 13, the LIBS spectra of *T. basicola* shows a lower calcium atom abundance as compared to the *M. phaseolina* LIBS spectra in Figure 14.The LIBS spectra of *M. phaseolina* also has a slightly higher abundance of magnesium atoms present as compared to the *T. basicola* spectra.

Overall, there is one key difference between the two species as shown by LIBS and one subtle difference. Unfortunately, multivariate statistical analysis was unable to be performed due to the small sample set. Though this does illustrate the promise LIBS has.

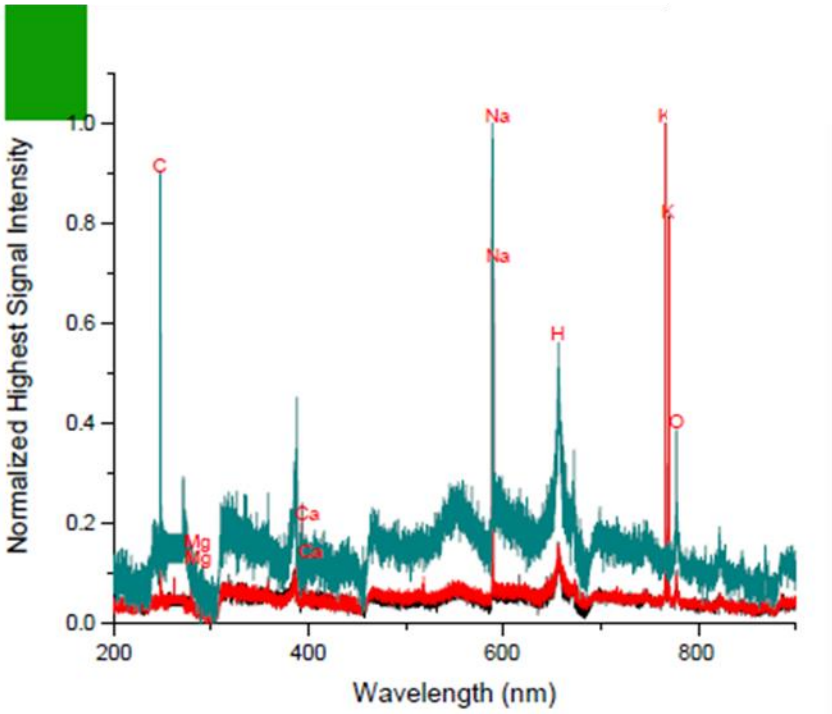

Figure 13 LIBS spectra of *T. basicola*

Two samples of *T. baiscola* are shown in the figure, the blue spectrum represents filter paper with no sample present, while the red shows the sample spectrum.

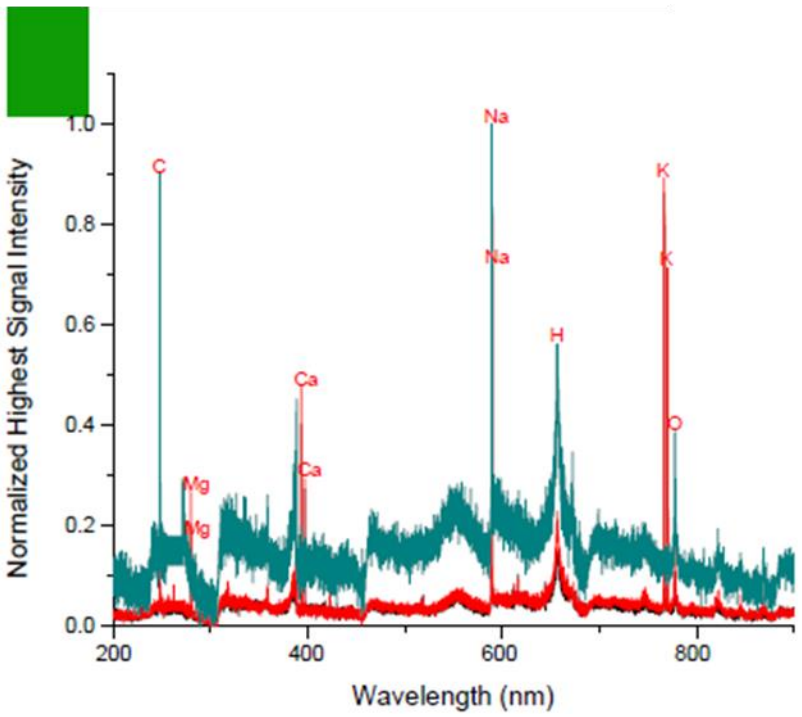

Figure 14 LIBS spectra of *M. phaseolina*.

Two samples of *M. phaseolina* are shown in the figure; the blue spectrum represents filter paper with no sample present, while the red shows the sample spectrum.

Conclusion

For the purpose of differentiating *M. phaseolina* and *T. basicola*, MALDI-TOF MS and FT-IR spectroscopy performed well and effectively differentiated the two species. While it was uncertain that LIBS did effectively differentiate the species, it was apparent that differences were present in the spectra. This shows a promising research point that could prove effective in the identification of filamentous fungi. Overall, MALDI-TOF MS and FT-IR spectroscopy data presented correlated with other research finding where the two techniques effectively differentiated *M. phaseolina* and *T. basicola*.[49,50]

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