Monitoring wheat mitochondrial compositional and respiratory changes using Fourier transform mid-infrared spectroscopy in response to agrochemical treatments

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ABSTRACT

Fungicides and plant growth regulators can impact plant growth outside of their effects on fungal pathogens. Although many of these chemicals are inhibitors of mitochondrial oxygen uptake, information remains limited as to whether they are able to modify other mitochondrial constituents. Fourier transform mid-infrared spectroscopy (FT-mIR) offers a high sample throughput method to comparatively and qualitatively evaluate the effects of exogenously added compounds on mitochondrial components. Therefore the objective of this study was to determine the ability of FT-mIR to detect effects mitochondrial fractions isolated from wheat (*Triticum aestivum* L.) seedlings in response to several agrochemical treatments, with an emphasis on fungicides. The accessed need was to develop FT-mIR analytical and statistical routines as an effective approach to differentiate spectra obtained from chemically-treated or untreated mitochondria. An NADH-dependent oxygen uptake approach was initially used as a comparative method to determine whether the fungicides (azoxystrobin, boscalid, cyazofamid, fluazinam, isopyrazam, and pyraclostrobin) and the plant growth regulator, (trinexapac-ethyl) reduced respiration inhibition on isolated mitochondria. Pyraclostrobin was the most effective inhibitor, whereas amisulbrom did not impact oxygen uptake. However, hierarchical clustering of FT-mIR spectra of isolated mitochondria treated with these different compounds separated into clades consistent with each of their expected mode of action. Analysis of the FT-mIR amide protein region indicated that amisulbrom and pyraclostrobin interacted with the isolated wheat mitochondria. Both chemicals were statistically different from the control signifying that respiration was indeed influenced by these treatments. Moreover, the entire FT-mIR region showed differences in various biological bands thereby providing additional information on mitochondria responses to agrochemicals, if so warranted.

Key Words: Fourier transform mid infrared; Mitochondria respiration; Fungicides; High-Throughput;

1. INTRODUCTION

Agrochemicals, such as fungicides, often have secondary activities in plants outside of their targeted pathogen or primary mode of action. Many fungicides are inhibitors of the highly conserved mitochondria electron transport proteins (FRAC, 2016). Strobilurins, a relatively new class of fungicide, which can target mitochondrial proteins, have been reported to positively impact cereal grain yields (Grossman & Retzlaff, 1997). However, the secondary effects of many agrochemicals on plant growth remains limited. Therefore, the objective of this study was to develop a FT-mIR method capable of obtaining biochemical information from chemically treated or untreated mitochondria as a means to classify agrochemicals based on their mode of cellular respiration inhibition.

Mid-Infrared (mIR) spectroscopy is considered a metabolic fingerprinting method that is able to simultaneously monitor carbohydrates, lipids, fatty acids, DNA, RNA, proteins, phosphate containing molecules and polysaccharides of intact cells, cellular organelles and other biological samples (Singh *et al.*, 2012, Payne and Veis, 1988, Alex and Dupuis, 1989, Hembram and Rao, 2009, Marty *et al.*, 2009, Kanakis *et al.*, 2009, Cherng, 2009, Ozek *et al.*, 2010, Anastassopoulou *et al.*, 2009, Banyay *et al.*, 2003, Froehlich *et al.*, 2011, Tallandier and Liquier, 1992). For a molecule to absorb IR light of a specific frequency (wavenumber $4000\text{-}200\text{ cm}^{-1}$), it must undergo a net change in dipole moment resulting in bond stretching, rocking and / or bending (Goormaghtigh *et al.*, 1999). Molecules can then be identified based on their mIR

fingerprint produced by these vibrations, which typically results in multiple and convoluted, bands representing one molecule.

Fourier transform mid infrared spectroscopy (FT-mIR) has been used to monitor plant physiological processes (Bertoluzza *et al.*, 1999, Ivanova and Singh, 2003). One such study showed that this technique was able to detect changes in the redox properties involved in oxidative phosphorylation (Marechal *et al.*, 2009). Here, the effectiveness of FT-mIR spectroscopy for detecting and classifying respiration inhibitor induced mitochondrial changes was evaluated by performing side-by-side studies with the NADH-dependent oxygen uptake assay. Eight chemicals were selected with a diverse range of target sites (Table 1). These chemicals were selected as they are commonly used as agricultural fungicides with the exception of trinexapac-ethyl, which is a plant growth regulator (FRAC, 2016, Heckman *et al.*, 2002). Based on the results, the FT-mIR approach was not only able to classify changes in mitochondrial function, but also was able discriminate between agrochemical-treated and untreated samples while providing additional spectral information of other potential biochemical changes.

2. MATERIALS AND METHODS

2.1 Mitochondria Isolation

Mitochondria were isolated from shoots of 3-day-old etiolated ‘Overland’ wheat seedlings using a modified Hanson (1972) procedure. Grinding media consisted of 350 mM mannitol, 30 mM 3-[N-morpholino]propane sulfonic acid (MOPS) and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.6. Following pH adjustment, 1.5 g of polyvinylpolypyrrolidone and 0.32 g L-cysteine were added to the grinding media. Centrifuge speeds were 5110 g for the debris

Table 1.

Common name	IUPAC Name	FRAC Code	Target Site
amisulbrom	3-(3-bromo-6-fluoro-2-methylindol-1-yl)sulfonyl-N,N-dimethyl-1,2,4-triazole-1-sulfonamide	C4	complex III: cytochrome bc1 at quinone inside site (FRAC, 2016)
azoxystrobin	methyl (E)-2-[2-[6-(2-cyanophenoxy)pyrimidin-4-yl]oxyphenyl]-3-methoxyprop-2-enoate	C3	complex III: cytochrome bc1 at quinone outside site (FRAC, 2016)
boscalid	2-chloro-N-[2-(4-chlorophenyl)phenyl]pyridine-3-carboxamide	C2	complex II: succinate-dehydrogenase (FRAC, 2016)
cyazofamid	4-chloro-2-cyano-N,N-dimethyl-5-(4-methylphenyl)imidazole-1-sulfonamide	C4	complex III: cytochrome bc1 at quinone inside site (FRAC, 2016)
fluazinam	3-chloro-N-[3-chloro-2,6-dinitro-4-(trifluoromethyl)phenyl]-5-(trifluoromethyl)pyridin-2-amine	C5	uncoupler of oxidative phosphorylation (FRAC, 2016)
isopyrazam	3-(Difluormethyl)-1-methyl- N - [1,2,3,4-tetrahydro-9-(1-methylethyl)-1,4-methanonaphthalen-5-yl]-1 H - pyrazol-4-carboxamid	C2	complex II: succinate-dehydrogenase (FRAC, 2016)
pyraclostrobin	methyl N-[2-[[1-(4-chlorophenyl)pyrazol-3-yl]oxymethyl]phenyl]-N-methoxycarbamate	C3	complex III: cytochrome bc1 at quinone outside site (FRAC, 2016)
trinexapac-ethyl	ethyl 4-[cyclopropyl(hydroxy)methylidene]-3,5-dioxocyclohexane-1-carboxylate		complex III: cytochrome bc1 (Heckman <i>et al.</i> , 2002)

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100 cleaning phase and 19,150 g for mitochondria collection. Higher centrifugation speeds were used
 101 to more efficiently clean and collect mitochondria. The pellet was washed and resuspended with
 102 media containing 300 mM mannitol, 20 mM MOPS and 1 mM EDTA, pH 7.2. For the final
 103 cleaning phase, the suspension was centrifuged at 5110 g for 2 min and the pellet was discarded.
 104 The final suspension media consisted of 250 mM sucrose and 30 mM MOPS, pH 7.2. Isolated

mitochondria intended for the NADH-dependent oxygen uptake assay were immediately stored at -80 °C until analysis. Isolated mitochondria to be used for FT-mIR analysis were diluted using the suspension media to an optical density of ~ 1.5 absorbance units (AU) at 600 nm to ensure high and consistent signal to noise ratios, and then immediately stored at -80 °C until analysis.

2.2 NADH-Dependent Oxygen Uptake

Isolated mitochondria were sonicated to disrupt membranes. Oxygen consumption was measured polarographically by using a Rank Brothers O electrode (Rank Brothers, Ltd., Cambridge, UK) at 25 °C in 1 mL of reaction medium containing 30 mM MOPS adjusted to pH 7.2. The oxygen content of air-saturated water was estimated according to Estabrook (1967). The reaction was initiated with 10 µL of 250 mM NADH. Mitochondria were treated with 1, 2.5, and 5 mM chemical treatments (Table 1) dissolved in solvent (DMSO, DCM or ethanol). Solvent controls were assessed to ensure all inhibition effects were specifically attributable to chemical treatments.

2.3 FT-mIR spectroscopy (sample preparation and data collection)

Isolated mitochondria were briefly thawed and divided into 100 µL aliquots. One microliter of 100, 250 or 500 mM inhibitor in solvent (DMSO, DCM, or ethanol) was dissolved in dimethyl sulfoxide (DMSO) and added to the mitochondrial solution to produce final treatment concentrations of 1, 2.5 and 5 mM. Treated mitochondria were lightly vortexed before plating. For FT-mIR analysis, 30 µL aliquots of treated and untreated mitochondrial preparations were pipetted onto a zinc selenide (ZnSe) plate and dried under vacuum (28 Hg) for 45 min at 35 °C. Chemical controls (treatments without mitochondria in MOPS) were also prepared and analyzed for use in subtraction procedures.

Temporal changes in the FT-mIR spectra of treated isolated mitochondria were evaluated against untreated mitochondria. Specifically, a Bruker Equinox 55 FT-MIR equipped with a deuterated triglycine sulfate detector was used to acquire FT-mIR spectra. Samples were applied to a ZnSe BioWheel, which contained 15 sample windows. A spectrum was recorded directly from a dried sample biofilm using the absorbance mode. Each spectrum was recorded in the mid IR range ($4000\text{-}500\text{ cm}^{-1}$) at a resolution of 4 cm^{-1} with 128 interferograms co-added, averaged and apodized with the Blackman-Harris 3-Term function and then Fourier-transformed.

Recording of spectra, data storage and all other spectral manipulations were performed using the Bruker Opus 4.0 Software. The spectra were independently and consistently baseline corrected, normalized to the amide I peak and Fourier self-deconvoluted before applying other spectral manipulations. A total of three biological replications with 3 technical replications per biological replication were collected and averaged to determine treatment means. Sample plating techniques followed a randomized complete block design, which were blocked for replicates. Averaged fungicidal control spectra were subtracted from the corresponding averaged mitochondrial treatments in the amide I region to ensure spectral differences reflected treatment-induced conformational shifts. Spectral areas were monitored in the amide I region ($\sim 1710\text{ - }1600\text{ cm}^{-1}$) and compared to the control through hierarchical cluster analysis (Ward's algorithm) and presented in the form of dendrograms (Ward, 1963).

2.4 Statistical analysis of FT-mIR spectral data

Further analysis of the FT-mIR spectra from amisulbrom, pyraclostrobin and the control groups was completed to determine statistically significant differences in the spectra between treatments. After the preprocessing described in Section 2.3, the spectra were imported into The Unscrambler X (version 10.3) software, and data tables were created for each zero order spectra

that were then converted to first derivative. Data tables were inputted to R statistical software (version 3.1.2), and a function was created to perform a student's T-test at each wavelength for comparing amisulbrom or pyraclostrobin treated samples to the untreated control similar to Malins *et al.* (2004). P-values were plotted at each wavelength using R software.

3. RESULTS AND DISCUSSION

3.1 NADH-dependent oxygen uptake assay

The NADH-dependent oxygen uptake results represent the oxygen consumption, or rate of respiration, of intact mitochondria or functional mitochondrial isolates. Lower values reflect increased respiration inhibition attributed to the corresponding chemical. No significant effect occurred between doses (data not shown), thus results for only the combined 1.0, 2.5 and 5.0

mM inhibitor concentrations are presented (Fig. 1). These results indicated that azoxystrobin and

Fig 1.

pyraclostrobin were the most effective inhibitors of mitochondrial respiration, as evidenced by the 62 and 55 percent inhibition to the controls, respectively. All of the remaining inhibitors, with the exception of amisulbrom, also significantly inhibited isolated wheat seedling mitochondrial respiration, but not to the extent as azoxystrobin and pyraclostrobin (Fig. 1). Although amisulbrom was not significantly different from the control, it is showing a downward trend.

3.2 FT-mIR Spectroscopy Amide Region Analysis

Because these inhibitors target proteins of the mitochondrial respiratory chain, initial analysis of the FT-mIR spectra focused on the amide protein region (Table 2). Hierarchical cluster analysis (HCA) (Malins *et al.*, 2004) was used to classify differences in FT-mIR obtained isolated and sonicated wheat seedling mitochondria were treated with the eight different oxidative phosphorylation inhibitors. A hierarchical cluster analysis of the inhibitors based on the data from the amide I protein region ($1700\text{-}1600\text{ cm}^{-1}$) is presented in the

form of a dendrogram (Fig. 2).

Fig. 2

Grouping of the inhibitor treated samples was consistent with their expected mode of inhibition of oxidative phosphorylation. While this method of analysis does not provide quantitative data on the inhibition of mitochondrial inhibition, determination of site of action of the respiration inhibitors is consistent with the previously established fungicide target sites (FRAC, 2016). This data (Fig. 2) also support the previously suggested secondary action of trinepach-ethyl as a cytochrome bc₁ inhibitor (Heckman *et al.*, 2002).

Amisulbrom and pyraclostrobin-treated and untreated control spectra were analyzed to further demonstrate the applicability of FT-mIR for detecting changes in respiration of isolated wheat seedling mitochondria induced by chemical treatments. These two chemicals were chosen based upon the NADH-dependent oxygen uptake data (Section 3.1). Amisulbrom did not inhibit respiration, whereas pyraclostrobin strongly inhibited respiration. By comparing the treated samples with their respective controls, the significance and biological detail of mitochondrial changes influenced by the inhibitor was examined. Mid infrared spectra of the dried films of

treated

Fig. 3

mitochondrial solutions contained noticeable differences in spectra representing biochemical changes between controls and fungicide-treated samples (Fig. 3). Spectra changes were evident in the zero order spectra of the amide I and II protein regions (1500-1800 cm^{-1}) as well as the mixed and fingerprint regions (800-1500 cm^{-1}) (Fig. 3) between the two fungicides and the untreated control. Changes in the amide protein regions (Table 2) may be indicative of oxidative phosphorylation protein interaction or conformational alteration after inhibitor treatment. Downstream response and changes in the spectra after metabolic intervention were also evident in the mixed and fingerprint region (Fig. 3).

Table 2.

Wavenumber (cm-1)	Assignment	Author
1659-1624	Amide I band heterogeneity groups in collagen triple helix	Vidal and Mello, 2011
~1656	Amide I	Zelig <i>et al.</i> , 2011
1651	Amide I	Anastassopoulou <i>et al.</i> , 2009
1640	Amide I polyproline II dried film	Lazarev <i>et al.</i> , 1985
1629	Amide I high H bonding; helical structure organization	Vidal and Mello, 2011
1549	Amide II stretching	Singh <i>et al.</i> , 2012
1547	Amide II	Anastassopoulou <i>et al.</i> , 2009
~1546	Amide II mode	Zelig <i>et al.</i> , 2011

As derivatives deconvolute the overlapping bands of zero order FT-mIR data, the first derivative of the untreated control, amisulbrom and pyraclostrobin spectra were also obtained in the amide protein region (Fig. 4). The differences in spectra are shown in the first derivative data, such as the leftward shift of pyraclostrobin in the amide II protein region at approximately

208 1550 cm^{-1} and the changes in spectra shape in the amide I protein region (1600-1700 cm^{-1}).
 209 These shifts indicate changes in the extracted mitochondria that occurred as a result of chemical

210 application. Because these compounds inhibit oxidative phosphorylation, it is plausible that the
 211 changes

212 **Fig 4.**

213 exhibited by the spectra are associated with the specific protein of the electron transport system.
 214 Subsequent multivariate analyses were next employed to classify and explain these differences.

215 Multivariate clustering methods is a powerful classifying tools for FT-mIR data
 216 (Goodacre *et al.*, 1998, Kümmerle *et al.*, 1998, Gonçalves *et al.*, 1998). However, such analyses
 217 lack statistical significance. To verify the power of FT-mIR to detect significant changes initially
 218 reported by the NADH-dependent oxygen uptake assay (Section 3.1), the Student's t-test was
 219 applied to each spectral wavelength as a means to compare the untreated control versus the
 220 fungicide treated groups. Figures plotting the p-values of the control versus amisulbrom or
 221 pyraclostrobin at each wavelength were then constructed (Fig. 5). Use of clustering methods in
 222 parallel with p-value plots provides information on the significance of the FT-mIR data and also

223 validates the clustering analysis (Malins *et al.*, 2003, Malins *et al.*, 2004). P-values beneath the
 224 dotted lines indicate significant difference at those wavelengths ($p < 0.1$ and $p < 0.05$). As
 225 expected, many regions of that were significantly different existed between the pyraclostrobin
 226 and the untreated control spectra (Fig 5B). Although the oxygen uptake assays failed to discern
 227 any difference between control and amisulbrom-treated mitochondria, the FT-mIR method
 228 indicated many regions were also significantly different between the amisulbrom and the
 229 untreated control spectra (Fig. 5A). These results not only provided statistical significance to the

230 dendrogram (Fig. 2), but more importantly,
 demonstrated that the FT-mIR method is able to
 detect treatment induced differences, which never
 be achieved by the NADH-dependent oxygen
 uptake assay (Section 3.1).

241 **Fig. 5.**

242 3.3 Additional FT-mIR regions of biological significance

While our investigation focused on the amide I protein band, many other biologically important regions existed within the mIR spectrum. Differences are apparent the entire FT-mIR regions represented in the first derivative spectrum of the untreated control and mitochondria treated with either amisulbrom or pyraclostrobin (Fig 6). These regions could be aligned with p-values (Fig. 7A and B) of the same wavenumbers to determine regions that merit further investigation. While only comparative analyses were used to show classification capability of treated mitochondria with FT-mIR, more detailed biological data could be extracted by reviewing the whole spectra. Biologically relevant peaks are present throughout the 800-1800 cm^{-1} wavenumber range (Table 2). Investigation by FT-mIR of isolated mitochondria could thus provide important biological information on the protein, fatty acid, DNA, lipid, glycogen or many other changes in samples in response to the different treatments relevant to the control.

Fig. 6.

Fig. 7.

While the aim of this study was to investigate the qualitative difference in isolated mitochondria, it is expected that by changing from transmission FT-mIR to FT-mIR-attenuated total reflectance (ATR) will allow spectra acquisition from crude samples, such as ground or

even whole leaves or other plant material. This approach will also allow for higher sampling speed and may provide a superior method for real time measurement of inferred chemical influence on mitochondrial function. Furthermore, mobile FT-mIR-ATR units exist (Bruker Optics, 2013), which could provide real time *in situ* measurement in the field research setting.

4. CONCLUSIONS

Mitochondria extracted from wheat (*Triticum aestivum* L.) were utilized to analyze the effectiveness of FT-mIR spectroscopy to discern changes occurring to mitochondrial components when treated with a variety of agrochemicals. The NADH-dependent oxygen uptake assay was used to quantitatively assess the extent of respiration inhibition between eight inhibitors and to select the least and most effective inhibitors for more detailed FT-mIR analyses. Data from the oxygen uptake assay indicated that amisulbrom was ineffective in reducing respiration whereas pyraclostrobin strongly reduced respiration. Examination of the amide I protein mIR region with HCA accurately classified the different inhibitors into clades that were consistent with their expected mode of inhibition of mitochondrial respiration. Significant differences in the FT-mIR spectra were obtained from both amisulbrom and pyraclostrobin-treated samples relative to the untreated control, indicating greater resolving power of the FT-mIR protocol. Results from this study provide a first glimpse of the wealth of biological data that can be extracted from FT-mIR spectra. This study also provides an impetus to apply FT-mIR as a means to understand non-targeted biological effects of exogenous chemicals on plant growth.

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395 **7. Table Titles and Figure Legends.**

396 **Table 1:** Common and IUPAC name of chemicals used in oxygen uptake experiments,
 397 fungicide resistance action used and target site for respiration.

Table 2. FT-mIR spectrum peak assignments for amide I and II protein ranges. Adapted from Mello and Vidal (2012).

Fig. 1. NADH-dependent oxygen uptake in mitochondria isolated from wheat seedlings in the presence or absence of respiration inhibitors. Data bars represent least significant means ($n=3$) $\pm$ standard error. Treatments with the same letter are not significantly different ($P \leq 0.05$) according to Tukey's HSD test.

Fig. 2. Cluster diagram of FT-mIR amide I protein region. Heterogeneity of treated isolated mitochondria as compared to untreated control mitochondria. Hierarchical cluster analysis was performed using Ward's algorithm in the protein (amide I) region (~ 1710 - 1600 cm^{-1}) of first derivative, vector normalized spectra. FRAC codes (Table 1) for site of action are shown in parentheses.

Fig. 3. FT-mIR zero order spectra of isolated mitochondria. Spectra are of the untreated control (solid line), amisulbrom (dashed line), or pyraclostrobin (dotted line) treated sonicated mitochondrial solution. Each spectrum is representative of three biological replicates, each with three technical replicates. Horizontal dotted lines bracket the wavenumber region used to prepare p-value figures and dendrogram analyses.

Fig. 4. FT-mIR first derivative spectra of amide protein region. Spectra are of the untreated control (solid line), amisulbrom (dashed line), or pyraclostrobin (dotted line) treated sonicated mitochondrial solution. Each spectrum is representative of three biological replicates, each with three technical replicates.

Fig. 5. FT-mIR amide I protein region p-value plots. Plots showing the t-test results (n=3) at each FT-mIR wavelength between the untreated control and amisulbrom (a) or pyraclostrobin (b). Horizontal dotted lines indicate p-values of 0.05 and 0.10.

Fig. 6. FT-mIR first derivative spectra. Spectra are of the untreated control (solid line), amisulbrom (dashed), or pyraclostrobin (dotted) treated sonicated mitochondrial solution. Each spectrum is representative of three biological replicates, each with three technical replicates (N=3).

Fig. 7. FT-mIR whole spectra p-value plots. Plots showing the t-test results (n=3) at each FT-mIR wavelength between the untreated control and amisulbrom (a) or the untreated control and pyraclostrobin (b). Horizontal dotted lines indicate p-values of 0.05 and 0.10.

GRAPHICAL ABSTRACT

Treat isolated mitochondria with fungicides.

FTIR spectra acquisition.

Determine statistically different regions in spectra of treated and untreated mitochondria.

Heirarchal cluster analysis of spectra.
Fungicides group by mode of action.