Project title:	Sensor based pre-symptomatic detection of pests and pathogens for precision scheduling of crop protection products
Project number:	CP119
Project leader:	Dr Martin McAinsh, Lancaster University Prof Frank Martin, University of Central Lancashire
Report:	Final report, 11, 2018 Year
Previous report:	Annual report, 2017
Key staff:	
Location of project:	Lancaster Environment Centre, Lancaster University
Industry Representative:	
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The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

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AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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CONTENTS

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GROWER SUMMARY

This project has not led to any definitive change in grower practices and has had no input from industry representatives and can therefore not be completed for this project.

Headline

There is potential for the development of vibrational spectroscopy as proximal sensor devices for the detection of abnormalities associated with pests and pathogens in crop plants. This project has demonstrated that microscopic pest detection is conceptually achievable both indirectly and directly pre- and post-harvest. In many cases, pre-visual symptomatic detection is possible. At this stage however, it remains difficult to quantify the financial benefits of the evaluated sensors, especially with respect to their current cost of operation for realistic commercial applications. Therefore, no change to growing practices based on this technology is currently beneficial.

Background

This project was intended to evaluate the feasibility of developing biospectroscopy (MIR and Raman spectroscopy) as sensors in various horticultural settings to mitigate crop loss. Rapid non-destructive sensors will aid in the development of sustainable technologies for the reduction of crop loss to pests and pathogens pre- and post-harvest. Early detection of pests and pathogens will increase the efficacy of crop protection products, while reducing their overuse and thus negative environmental impacts.

Summary

The exploratory nature of the project and preliminary results generated have not been reproduced in practical growing situations. Nevertheless, autonomous detection of disease at various points of progression from pre-symptomatic to late symptomatic, based on spectral data obtained by MIR spectrometers has shown high accuracy rates. In the context of this research and the specific methods used, preliminary detection rates range from around 80-100% accuracy. Higher accuracy is achieved for direct detection compared to indirect detection of pathogens. If such results can be translated into horticultural practice, there is significant potential to detect pathogens and reduce crop loss autonomously through incorporation into horticultural machinery or alternatively hand-held sensors.

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Financial Benefits

As no consultation with industry representatives was undertaken throughout this project, an adequate financial benefits analysis has not been performed.

Action Points

- In conclusion of this project, no change to grower practice is advised.
- When these sensors become commercially available, training and/or consultation will be required.

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SCIENCE SECTION

Introduction

Food security is becoming ever more challenging due to rapid global population rise, decreasing natural resources, and increasingly variable growing conditions due to climate change. Decreased horticultural productivity in the form of pre- and post-harvest crop loss caused by unfavourable environmental conditions, pests, and pathogens, threatens food security. The numbers of variables involved in modern agriculture, specifically with regard to mitigating loss are impressive as well as challenging. Crop loss occurs in both pre- and post-harvest stages with further loss incurred at virtually all points along the food supply chain (harvest process, packaging, distribution, consumer waste etc.). Weather, pests and pathogens, besides consumer waste within the food production/food supply chains, contribute to the large amount of overall loss within the annual production cycle. The amount of loss annually, although similar in developed and developing nations, is caused by different factors but may be as much as 40% (Gerland et al. 2014). Problems associated with crop loss include economic losses, global malnourishment, natural resource depletion, and excessive use of crop protection products that have negative environmental impacts (Savary et al. 2012). Considering extensive projected deficits in major crop production in the future (Ray et al. 2013), solutions must be found to reduce the amount of crop loss in pre- and post-harvest settings.

The large number of variables in horticulture makes the development of methods for sustainable crop protection difficult. Modern farming already employs numerous sensor technologies for crop monitoring. From this it is clear that no single sensor technology will be able to meet all the demands required by precision farming, reflected by the plethora of sensors currently being developed (Sankaran et al. 2010; Mahlein 2016). Especially the complex biology of pests is challenging to predict and has hindered the development of commercially available tools which are easy to use to detect pests and related disease (Mahlein 2016). Nonetheless, growers have access to a variety of pest control options aimed at plant disease identification and/or quantification (Martinelli et al. 2014). Drawbacks are that many of these options are destructive to plants, laborious, and difficult to use, or require extensive training and expertise. These drawbacks lead to the subjective evaluation of disease states by growers in many cases. Subjective performance can be highly variable, especially prior to onset of visual symptoms detectable by eye (Mahlein et al. 2012). Providing growers and perhaps non-experts with tools to detect, identify, and even quantify disease, damage, or other parameters relevant to quality assurance, thus appear to be a high priority for the future. A sustainable agricultural framework will therefore require an approach that

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incorporates effective and high throughput sensor technologies adaptable to an annual growing cycle (or plant life cycle), from crop to crop, and throughout potentially highly heterogeneous field settings (Skolik et al. 2018). Sustainable farming will require an approach that incorporates technologies able to detect negative effects, of organisms (pests) and the environment, on plants and harvested products in a non-destructive manner. Various non-destructive optical sensors are therefore candidate technologies for the mitigation of crop loss within horticultural systems (Mahlein 2016). Ideally, sensor information may be used for both predictions, such as real time decision making in agricultural systems, while simultaneously contributing to our knowledge of plant function on the many levels of biological organization. Adapting sensor technologies for direct application, while augmenting the comprehension of the associated biological processes, fits well within a sustainable framework.

Vibrational spectroscopy in biology (biospectroscopy) has been successful as a biomolecular sensor, and extensively used for, disease detection and discrimination of abnormal cells and tissues based on spectral alterations. Biospectroscopy has become an established field within the past few decades covering studies in molecular and cell biology, tissue analysis of sentinel organisms to track persistent pollutants, environmental monitoring including whole plant analysis (Butler et al. 2015). These new applications of vibrational spectroscopy have prompted the evaluation of this technology as a non-destructive sensor in plant science leading to horticultural applications. Biospectroscopy experiments involve sample preparation, spectral acquisition, and data analysis. Vibrational methods including Mid-Infrared (MIR) and Raman spectroscopy are among the most well-known spectroscopies for investigating biological materials including cells and tissues (Chan and Kazarian 2015). Biospectroscopy generates an information rich spectrum, indicative of chemical constituents for multi-component analysis. MIR and Raman spectroscopy employ light between 2.5-25µm wavelengths to excite the energy of molecular arrangements, where MIR light energy corresponds to vibrational and rotational modes of biochemical functional groups present in proteins, lipids, carbohydrates, and nucleic acids (Baker et al. 2014; Kazarian and Chan 2013). MIR and Raman provide complementary information on the relative abundance and types of chemical structures within a sample, in the form of a wavenumber spectrum (Figure 1).

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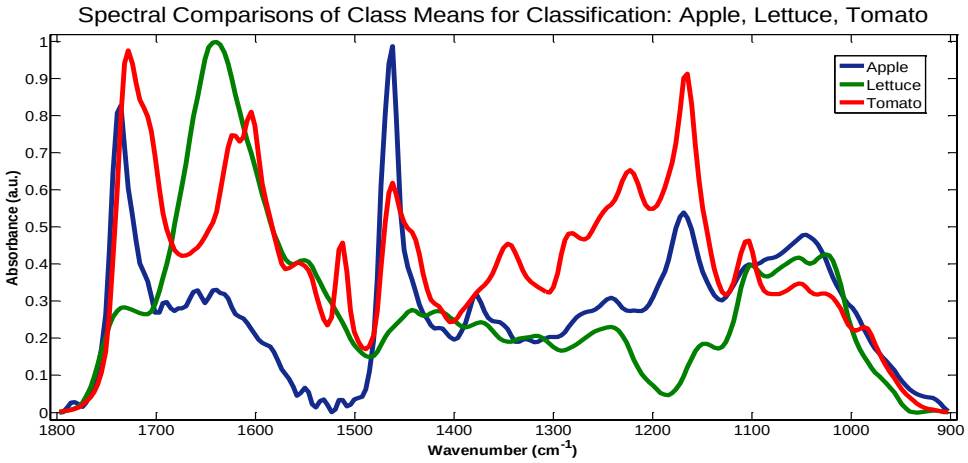

Figure 1: Attenuated total reflectance Fourier transform infrared (ATR-FTIR) class mean spectra of apple peel (blue), lettuce leaves (green), and tomato skin (red) over the 'fingerprint' region (1800-900cm^{-1}) of the MIR range.

Modern MIR spectroscopy generates highly characteristic spectra based on light absorption patterns of a sample producing a characteristic spectral 'fingerprint' or 'signature' and represent a collection of hundreds of potential chemical bonds (Butler et al. 2016). High dimensional spectral data allows the extraction of useful disease markers or biomarkers associated with an abnormal state (damage, disease, contamination, deterioration, etc.). Spectral alterations serving as such 'biomarkers' are generally extracted as part of an exploratory framework, after which these may be evaluated as part of a diagnostic framework aimed at making autonomous predictions as part of a machine learning approach (Trevisan et al. 2012). For horticultural applications, the accuracy of a diagnostic framework must be sufficiently high before it can serve as an automated decision-making platform in high throughput systems. Promising decision-making platforms are initially designed and tested, then used and refined. Although diagnostic frameworks may supplement expert evaluations when needed, autonomous predictions, even in the absence of experts, will be an increasing necessity as part of sustainable practices. Raman spectroscopy has been extensively used for direct plant disease detection (Egging et al. 2018; Faber and Kurouski 2018). Literature and protocols reviewing biospectroscopy approaches for (first time) users have recently become established, shown by the commitment to bring these technologies into practice (Baker et al. 2014; Butler et al. 2016; Martin et al. 2010). These provide the necessary information for user friendly applications on a small scale, or large-scale application in industry research and development. Therefore, it seems that the rapid advancement of the field of biospectroscopy and the number of scientific disciplines coming together as part of this endeavour, through knowledge transfer, has warranted a closer look at the potential applications as sensors in plant biology and agriculture (Skolik et al. 2018).

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This PhD project is aimed at evaluating MIR biospectroscopy as a potential solution, through the pre-symptomatic detection of pests and pathogens to reduce crop loss. By evaluating MIR methods, knowledge transfer is applied to bridge biospectroscopy with precision crop protection through the development of rapid sensors. We aim to demonstrate that MIR biospectroscopy can contribute to reduce crop loss by early pest detection leading to a more precise intervention with crop protection measures, to reduce both adverse effects on plants and crops, limit disease transmission, while reducing the negative effects of excess pesticides in the environment. Additionally, the monitoring of produce shelf-life, deterioration, adulteration, ripening, and ageing is part of the project. This project will help identify major problem areas to address in both the pre- and post-harvest stages of the food production and supply and give researchers together with industry partners a foundation on which to build collaboration for the future.

Materials and methods

Plant Growth Conditions

Materials and methods for this PhD project differ slightly with regard to plant cultivation conditions, subjects, and treatments across the numerous experiments performed. Glasshouse conditions for experiments with *Solanum lycopersicum* cv. Moneymaker (Thompson and Morgan Seeds, UK) were typically set at the following conditions: 18/6hr photoperiod with irradiance between 200-500 µmol/m^2/s; 30-50% relative humidity; Levington's M3 growth medium; in 1L (13cm) or 2L (17cm) pots. Plants were watered once daily to soil holding capacity. Tomato fruits were analysed from commercial stores or harvested from plants grown in the same conditions as mentioned above but in 20L pots to fruit maturity. Survey experiments were conducted on apple, lettuce, and pepper to determine whether detailed experiments performed on tomato (model system for this research) could be transferred to other crops.

Botrytis cinerea - Inoculum Preparation and Infection of Plants

Plants were inoculated with a suspension of spores of *B. cinerea* according to Asselbergh et al. (2007) and optimized for plants grown under the specified glasshouse conditions. Frozen (-80°C) 8 mL stock plugs of potato dextrose agar (PDA) containing mycelium of *B. cinerea* [strain R16] (Faretra and Palestra 1991), were placed in the centre of petri dishes containing freshly prepared PDA medium. The Petri dishes were incubated in a dark growth chamber (Percival AR-36L3) at 22 °C and 100 % humidity for 5 days until complete mycelial coverage of the PDA medium, after which they were exposed to a near-UV (UVA; 350-500 nm) light cycle (12 h dark/light; intensity: 28 µmol m^{-2} s^{-1}; bulb: L 18 W/77, Osram Fluora)for 7 days to induce sporulation (Schumacher 2017). Loose spores were washed into a 50 mL falcon tube,

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using approx. 15 mL deionized water. The remaining PDA plate containing mycelium and spores was cut into pieces using a sterile scalpel and added to a separate falcon tube containing 0.01 % Tween-20 (Polyoxyethylenesorbitan monolaurate) in 20 mL deionized water, and subsequently vortexed for 3 min. After intense mixing, the solution was gravity filtered through double-layered 20 µm nylon mesh into a 50 mL falcon tube to separate spores from mycelial debris and PDA medium. Filtrate containing spores in 0.01 % aqueous Tween-20, as well as the separate loose spores, were centrifuged for 15 min at 10,000 rpm at 15 °C. Supernatant was removed by decanting and spores were re-suspended and combined in 15 mL molecular grade water (Sigma Aldrich, UK). The concentration of spores determined via hemocytometer was adjusted to $5x10^5$ spores/mL using freshly prepared 0.1 M KH_2PO_4 and glucose solutions to give final concentrations of 0.05 M and 33 mM, respectively (Asselbergh et al. 2007). This solution was prepared three hours before application to allow pre-germination of spores, prior to infection of plants. Individual tomato plants were briefly removed from the greenhouse, placed into a containment area where only the shoot was exposed, and uniformly sprayed from above with approx. 1 cm^3 of spore solution at approx. 45° from plant canopy at 20 cm distance. This was repeated four times rotating plants 45° after each. Control plants were treated with a mock solution containing only 0.05 M glucose, 33.3 mM KH_2PO_4, and no spores. Following inoculation, plans were returned to the glasshouse to promote infection. Humidity was maintained at 100 % for 24 h using a glasshouse mister (Easy Irrigation, UK) combined with water timer (Easy-Control 1882, Gardena, UK) producing a 50 µm spray for 15 min every 2 h. Twenty plants were used for each treatment (20 mock/20 infected); 19 out of 20 plants infected developed symptoms, while the 20 mock controls remained asymptomatic throughout the experiment. Eight plants from each treatment (mock and infected) were reserved to confirm symptom development independent of ATR-FTIR analysis. Symptoms at the three measured time points, summarized in Table 1, were consistent with various stages of infection as previously described (Asselbergh et al. 2007; Audenaert et al. 2002; El Oirdi et al. 2011).

Sample Preparation

To simulate field conditions and realistic post-harvest scenarios, as well as to facilitate entirely non-destructive measurements, minimal sample preparation was performed. Apart from very few experiments, all samples (plants and fruit) were analysed intact. Typically, de-ionized water and clean cloth was used to remove dust, dirt, and obvious debris from fruit prior to spectral analysis as these interfere with the laser light incident on the fruit/vegetable.

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Spectral Acquisition

FTIR spectra were obtained with either a benchtop Bruker TENSOR 27, or semi-portable Bruker ALPHA-P FTIR, spectrometer with Attenuated Total Reflectance (ATR) module (Bruker Optics, Germany). Parameters were a sampling area of 250 by 250 µm or 1 mm^2 for the TENSOR and ALPHA respectively. Spectra were taken at a spectral resolution of 8 cm^{-1} (3.84 cm^{-1} data spacing), with 32 co-additions and a mirror velocity of 2.2/7.5 kHz for optimum signal to noise ratio (Martin et al. 2010). Background spectra were taken prior to each new sample, to account for any changes in atmospheric conditions. The ATR diamond was cleaned with Bruker ATR cleaning wipes prior to new sample measurement to ensure no spectral contributions of previous substances remaining on the diamond which are not water soluble. The penetration depth of the ATR-FTIR evanescent wave ranges between 0.5 and 5 µm at 4000–700cm^{-1}. Raman analysis was not employed during year 2 of this project as the FTIR spectrometers clearly showed better application potential with regard to the aims and objectives of this PhD research.

Data Analysis

Dataset analysis was conducted using the open-source IRootLab toolbox (http://trevisanj.github.io/irootlab/) (Trevisan et al. 2013) combined with Matlab 2014a (The Maths Works, MA, USA). Raw ATR-FTIR spectra were cut at the spectral fingerprint region, 1800–900 cm^{-1}, as this is where biological molecules predominantly absorb. Analysis steps in sequence involved pre-processing, normalization, PCA analysis, PCA-LDA analysis, and wavenumber extraction through either a cluster vector (CV) or loadings approach combined with a peak picking algorithm (16 cm^{-1} and 5 peaks / 8 cm^{-1} and 10 peaks) previously (for detailed background information on data analysis see Trevisan et al. 2012). Large spectral datasets contain hundreds of data points which undergo pre-processing, standardization / normalization, and feature extraction by way of data reduction. Unsupervised and supervised data analysis including principal component analysis (PCA) and linear discriminant analysis (LDA) respectively, are effective as part of exploratory analysis. Data reduction and feature extraction using PCA and or PCA-LDA permits the differentiation of dataset variance where PCA allows the visualization and extraction of features (principal components) responsible for overall dataset variance including intra-class variance. Intra-class variance, analogous to natural heterogeneity, may be more predominant than differences attributed to the assigned class or category (i.e. control vs treatment 1 vs treatment 2 etc.). By combining PCA with LDA (PCA-LDA), it is possible to maximize class or categorical variance, while minimizing the contributions from overall dataset variance. As part of the project was the use of classifier algorithms as part of a diagnostic (predictive) framework. These classifiers, including support vector machine (SVM) and linear discriminant classifier (LDC), evaluate how well MIR spectra

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serve to identify class treatment (normal from abnormal) within autonomous computer systems.

In summary, the exploratory framework employing PCA and LDA investigate the biological basis (changes in the biochemistry) for spectral changes, while classifiers use the spectral matrix (x/y number matrix) to determine how well these variables can be used to predict a specific condition. Combined this gives biological insight, as well as information on sensor performance for direct commercial application.

Results

This PhD research has demonstrated that the continued development of MIR biospectroscopy methods in the plant and crop sciences has strong potential to be applied in realistic horticultural scenarios to prevent crop loss associated with pests and pathogens. As a sensor-based technology it was shown that in many instances, pre-symptomatic detection of pests is achievable in the laboratory and plausible in the field with the specific spectrometers used. An ability to detect pathogens pre-symptomatically fulfils the criteria for the theoretical application to precision scheduled application of crop protection products. Benefits, theoretical, and practical applications of MIR spectroscopy for fundamental plant science and crop research are summarized by Skolik et al. (2018) and give an overview of the fundamental aspects pertaining to this PhD project work.

Presented results are a culmination of experiments included in the PhD research and represent the main conclusions of the project. These results therefore do not include range finding experiments, which are included in the annual report 2016 but not specifically relevant for the results and conclusions in the final report 2018. Results reported in the annual report 2017 are encompassed and expanded here as part of the final report 2018 for AHDB project CP119.

Determination of Developmental and Ripening Stages of Whole Tomato Fruit using Infrared Spectroscopy and Chemometrics

To set a baseline spectrochemical characterization of tomato fruit prior to the investigation of pathogen effects, a development and ripening study was performed to determine the effectiveness of MIR biospectroscopy to monitor healthy tomato fruit development and ripening (Figure 2).

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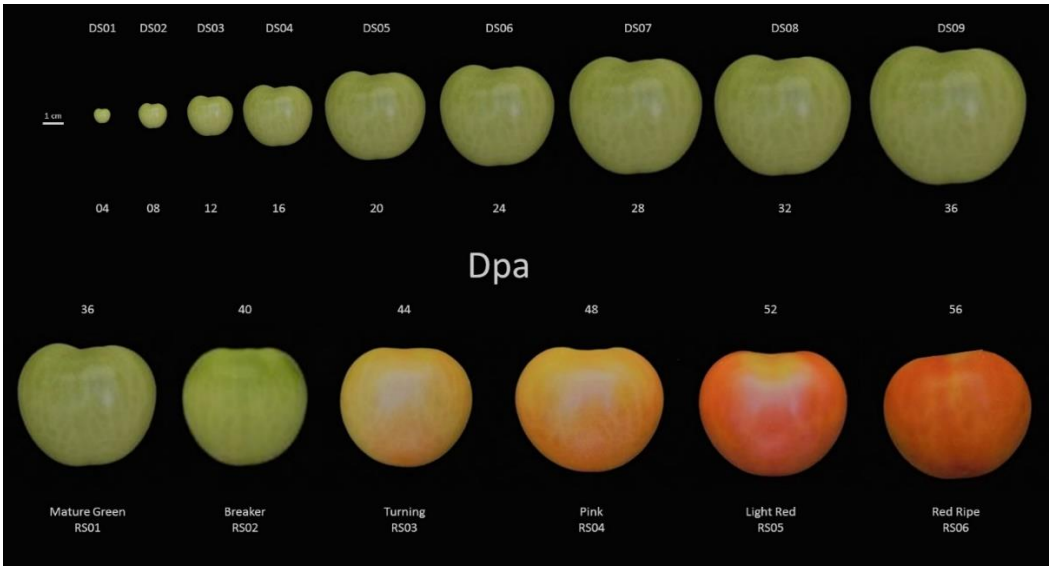

Figure 2: Tomato fruit of *Solanum lycopersicum* cv. Moneymaker: developmental (top) and ripening (bottom) stages used as individual groups for MIR ATR-FTIR analysis; dpa (days post-anthesis).

ATR-FTIR spectroscopy can measure large groups of compounds in epidermal structures of whole tomato fruit to determine development and ripening status. Exploratory discriminant analysis (PCA-LDA) associated these groups of compounds with specific biomarkers of tomato fruit development and ripening, identifying both common and unique spectral features reflecting the distinct changes occurring during tomato fruit development and ripening. The various compounds reflected by the fingerprint spectra can be tentatively assigned to components from epidermal surface layers including the cuticle and cell wall. As part of the intact cutinized cell wall, compounds including Cutin, waxes, phenolics, cellulose, pectin, and lignin were present, which showed major alterations although qualitative interpretation of spectral biomarkers remains challenging due to limitations in our knowledge of how the cell wall-cuticle complex changes during fruit development (Dominguez et al. 2015; Segado et al. 2015). Nevertheless, epidermal layers play important roles in the quality of fruit, as well as in the determination of horticultural grades at various points of tomato fruit development (Figure 3 and 4) (Lara et al. 2014). Automatic grading of the defined tomato fruit groups was evaluated using the SVM classification model (Table 1) indicating that development and ripening (Table 2) can be distinguished at a minimum of 15 separate stages (9 for development and 6 for ripening). Importantly, all analyses were entirely non-destructive and were performed using a compact portable ATR-FTIR spectrometer suggesting the potential for field-based analysis.

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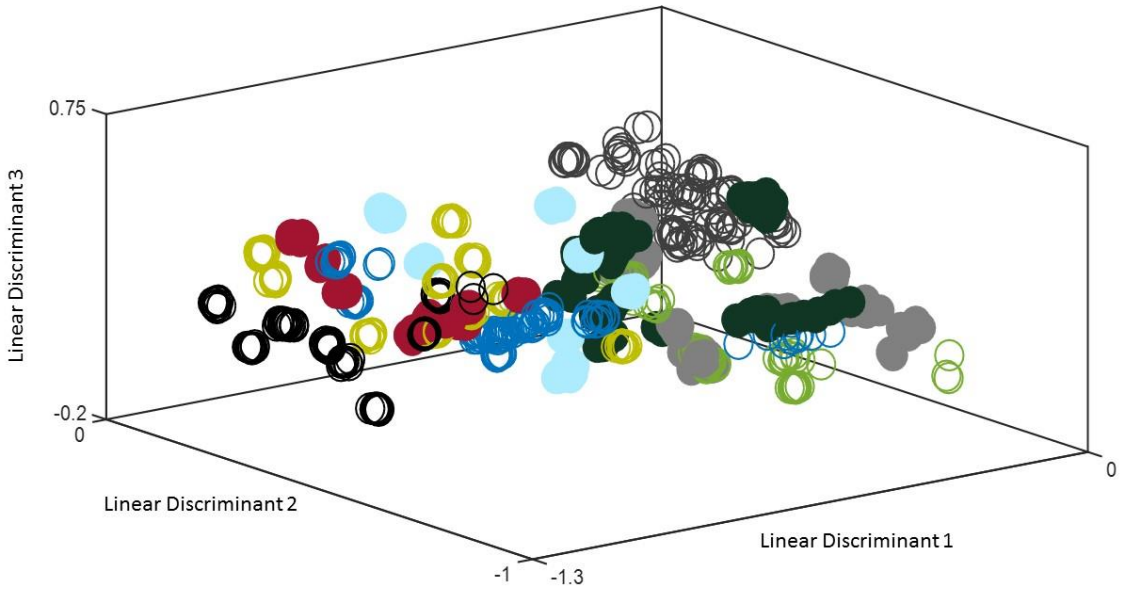

Figure 3: PCA-LDA 3-dimensional scores plot of tomato fruit developmental stages (DS01: dark grey circles; DS02: light grey; DS03: light green circles; DS04: dark green; DS05: blue circles; DS06: light blue; DS07: yellow circles; DS08: Purple; DS09: black circles).

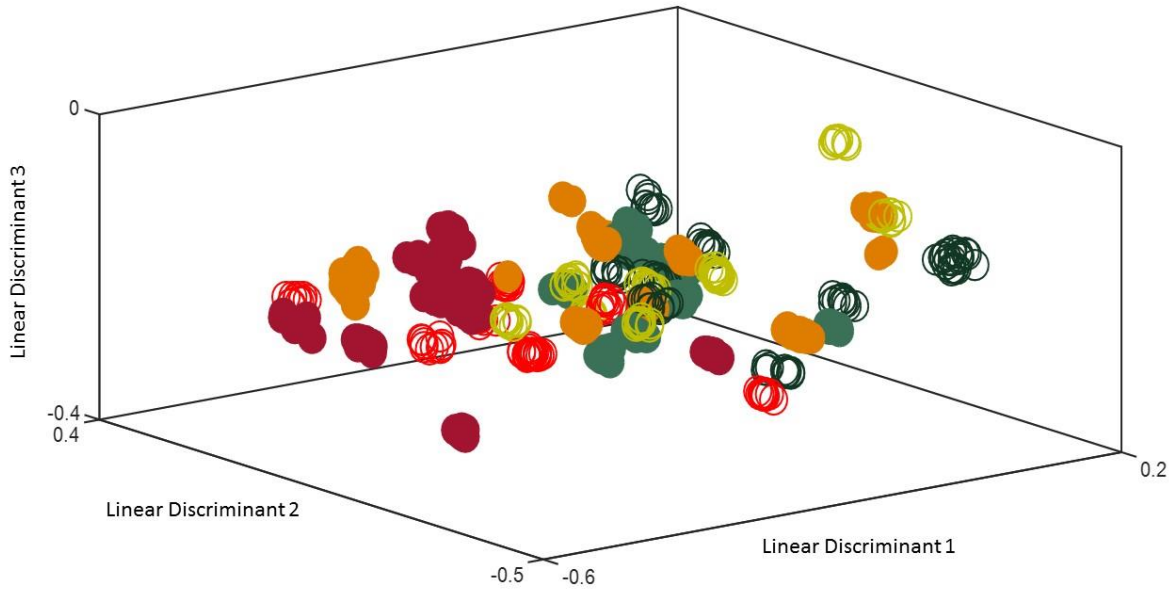

Figure 4: PCA-LDA 3-dimensional scores plot of tomato fruit ripening stages (RS01: dark green circles; RS02: green; RS03: yellow circles; RS04: orange; RS05: red circles; RS06: dark red).

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Table 1: Predictive performance presented as sensitivity and specificity rates calculated for the SVM chemo-metric model intended to differentiate tomato fruit developmental stages from their ATR-FTIR spectral data.

Developmental Stage (dpa)	Sensitivity	Specificity
DS01 (04)	100%	100%
DS02 (08)	100%	100%
DS03 (12)	100%	100%
DS04 (16)	100%	100%
DS05 (20)	100%	100%
DS06 (24)	100%	100%
DS07 (28)	100%	100%
DS08 (32)	100%	99%
DS09 (36)	99%	100%

Table 2: Predictive performance presented as sensitivity and specificity rates calculated for the SVM chemo-metric model intended to differentiate tomato fruit ripening stages from their ATR-FTIR spectral data.

Ripening Stage	Sensitivity	Specificity
Mature Green	100%	100%
Breaker	100%	100%
Turning	100%	99%
Pink	99%	100%
Light Red	100%	100%
Red	100%	100%

Most elements needed to transition this approach from a lab-based analytical method to an applied sensor technology for routine monitoring are already available including portable spectrometers, fast data analysis tools, and the minimal to no sample preparation required for most crop plants making this a realistic possibility. To realise this potential, application of biospectroscopy to additional model plant systems is needed alongside the evaluation of new portable equipment, like that recently developed for Raman spectroscopy (Farber and Kurouski 2018). With these advances, rapid analysis with optical sensors such as MIR spectroscopy will further permit the automatic characterization of healthy fruit development, and enabling abnormalities related to damage or disease to be reliably identified. In addition,

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further development of biospectroscopy in the plant and crop sciences will contribute to a better biological and biochemical understanding of plant surface layers, and how these affect the traits of plant organs such as fruit; thereby, contributing to both molecular plant biology and industrial horticulture for better crop production.

ATR-FTIR Spectroscopy Non-Destructively Detects Damage-Induced Sour Rot Infection in Whole Tomato Fruit

Multivariate analysis (PCA-LDA) can effectively discriminate healthy and compromised tomato fruit (Figure 5), based on damage and sour rot infection by *G. candidum*, effectively detecting pathogens indirectly. Spectral alterations in tomato fruit epidermis caused by damage and sour rot, induced changes in cuticle structure, which were assigned as tentative biomarkers. Damage, early and late stage infected fruit thus showed unique spectral profiles (Figure 6), while partial overlap of spectral markers between damage and early infection, as well as damage and late infection suggests a potential for disease specificity at these distinct stages.

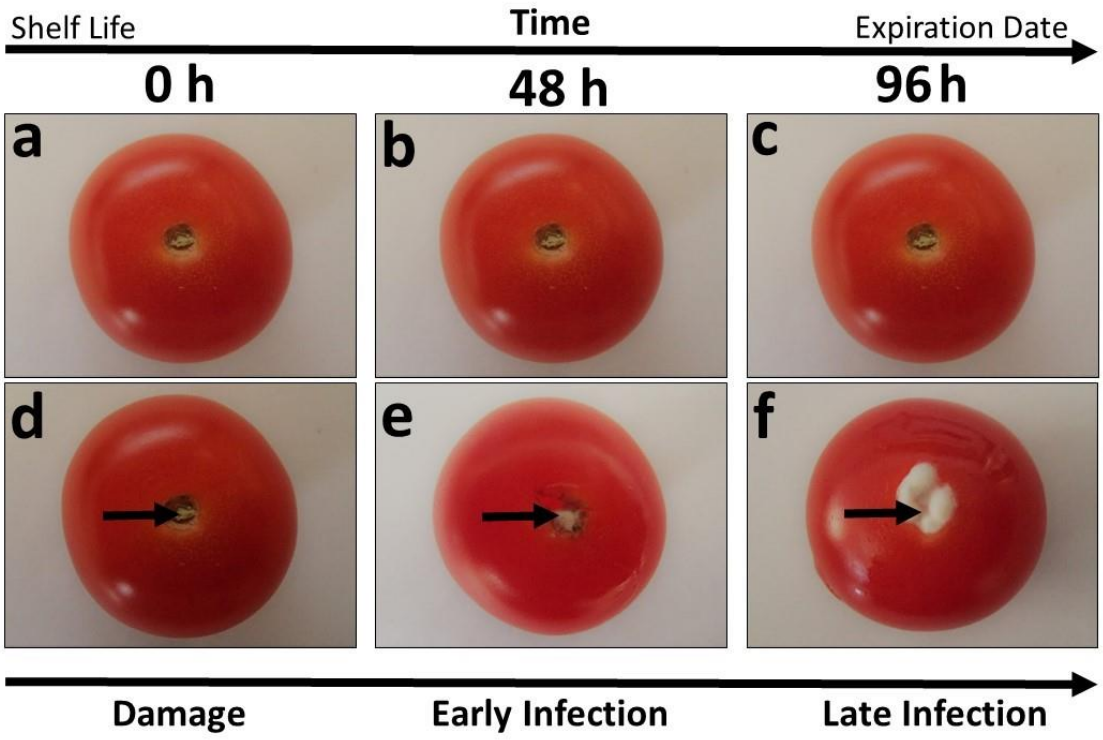

Figure 5: Symptoms associated with tomato fruit damage (d), early (e), and late (f) infection of tomato fruit by *G. candidum* compared to their shelf-life matched controls (a-c).

Disease specificity based on unique spectral markers is tentatively linked to complex and evolving stress responses. While the exact connection between spectral biomarkers of

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compromised tomato fruit and specific stress responses remains unclear, they are linked either directly or indirectly to plant responses such as ROS, SAR, and the HR. Clear alterations observed between healthy and damaged tomatoes further suggests the potential to identify damaged fruit prior to pathogen colonization. This may prevent disease spread, or to repurpose unmarketable specimens. Spectra of fungal pathogens and tomato fruit are fundamentally different offering direct detection of colonized pathogens within the intact fruit-pathogen complex.

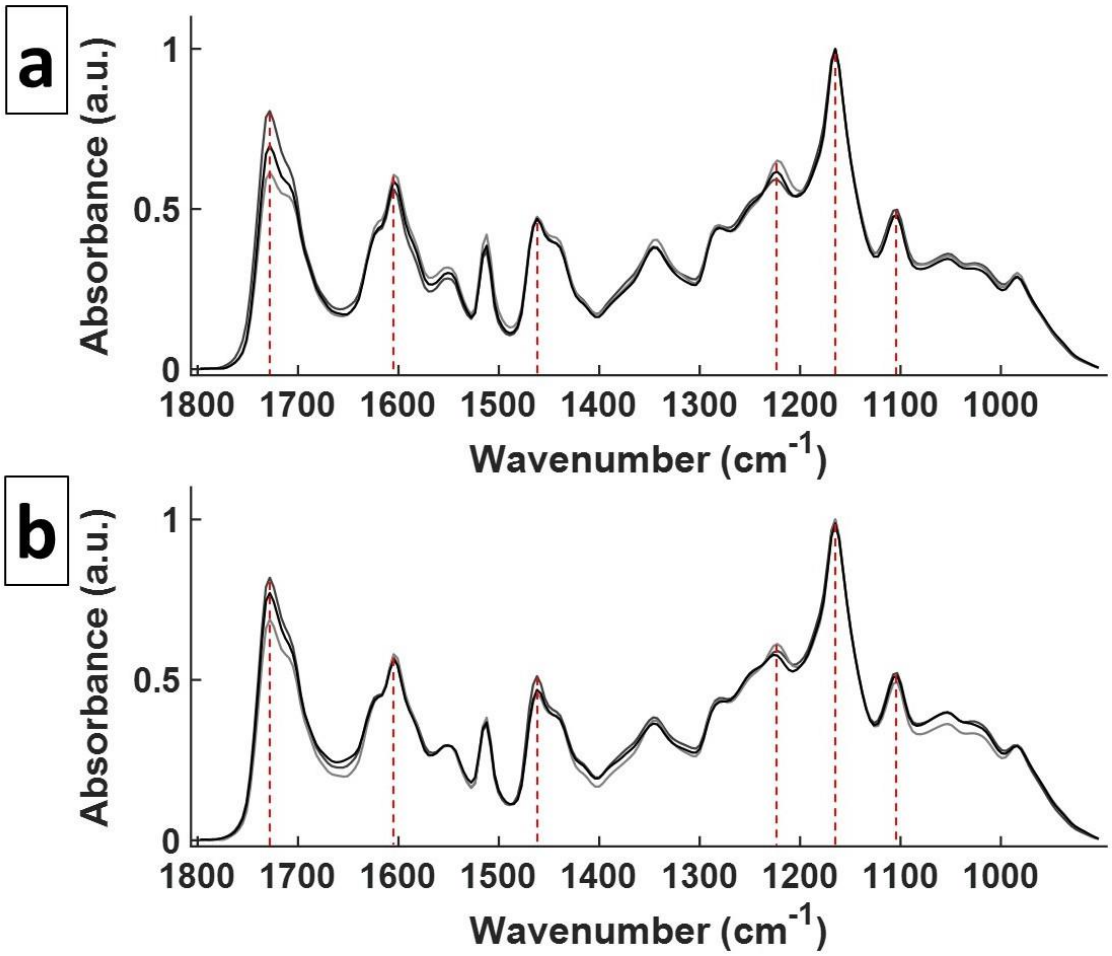

Figure 6: ATR-FTIR spectrum of intact tomato fruit *S. Lycopersicum* cv. Piccolo, over the fingerprint region (1800-900 cm^{-1}); **a** control series at 0 (light grey), 48 (grey), and 96 (black) h; **b** 0 h damaged (light grey), 48 h early infection (grey), and 96 h late infection (black).

Automatic detection of damage, early, and late infection through changes in fruit epidermal surface layers was evaluated based on the related classification model PCA-LDC. Indirect detection of damage and infection was shown to be effective with detection accuracy improving with disease development (Figure 7). Classification of tomato fruit damage and

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infection may be improved through knowledge transfer, the use of more sophisticated classification models, and trials with larger sample cohorts available to commercial growers.

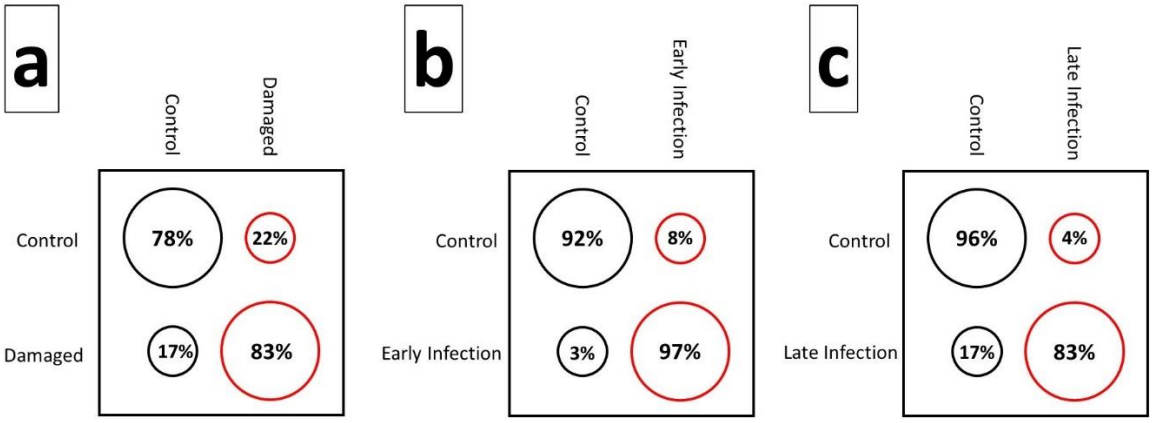

Figure 7: Classification rates (%) of damage, early, and late infection, compared with shelf-life matched controls, extracted from PCA input to linear discriminant classifier (PCA-LDC).

Adapting spectrochemical analysis for fundamental plant science has been successful, yet more work is required to exploit the sensor potential of MIR spectrochemical analysis in complex crop systems. Herein, we demonstrate the ability to analyse individual parts of plant-pathogen complexes *in vivo* and show that effects of damage and infection generate unique spectral signatures reflecting common stress responses in fruits. These signatures are effective for the autonomous detection of compromised fruit crops non-destructively and both direct and indirect detection of fruit pathogens. This opens the door for future work, which may focus increasingly on intact or native plant systems. Portable spectrochemical analysis equipment including MIR and Raman probes are becoming increasingly available and just beginning to be explored for crop analysis (Egging et al. 2018; Farber and Kurouski 2018; Fu et al. 2016; Trebolazabala et al. 2013; Yeturu et al. 2016). Rapid developments in MIR spectrochemical analysis for plant and crop science, will likely to lead to concrete large-scale applications for crop protection and production.

Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy-Coupled Chemometrics Directly Detects Pre- and Post-Symptomatic Changes in Tomato Plants Infected with Botrytis cinerea

Biospectroscopy presents a novel analytical technique yet has been minimally used in the plant and crop sciences, especially for applications to dynamic intact plant-pathogen systems (Skolik et al. 2018). Here it was demonstrated that biospectroscopy in the form of semi-

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portable ATR-FTIR was effective at non-destructive *in vivo* analysis of plant-pathogen interaction between *B. cinerea* and *S. lycopersicum* at the whole-plant level (Figure 8).

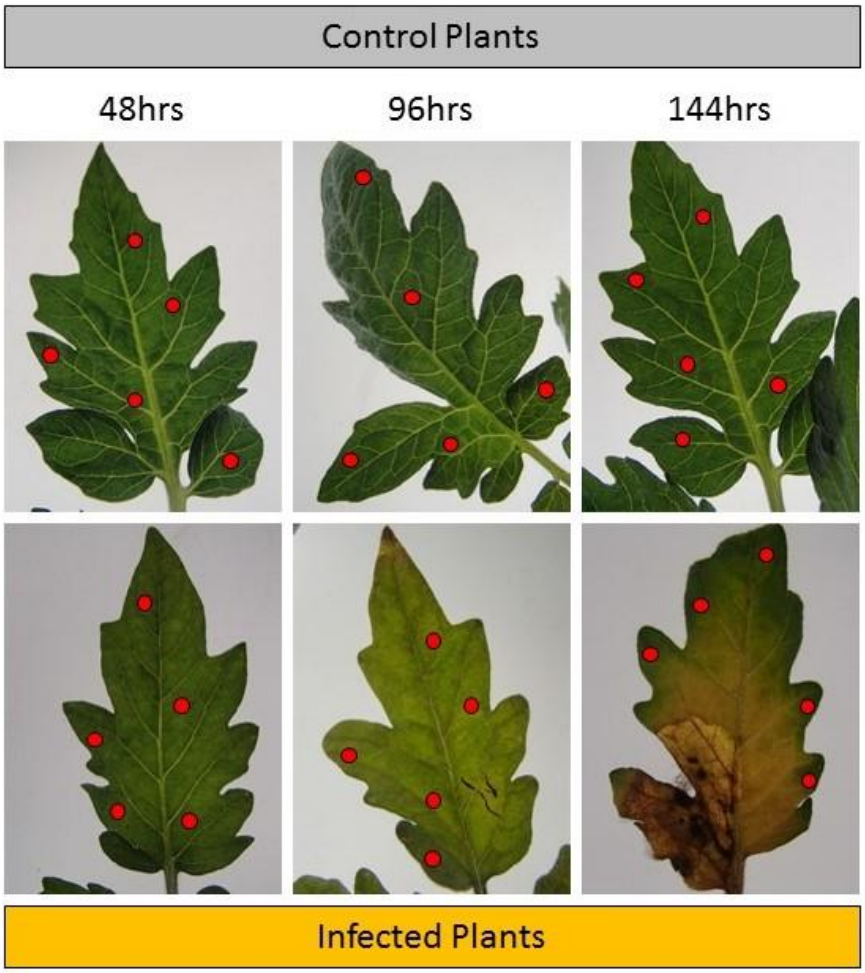

Figure 8: Examples of spectral acquisition points on leaves of whole tomato plants. Measurements were taken from healthy looking tissue of infected leaves as shown (this was only necessary in symptomatic tissue (infected leaves at 96 and 144 h Post Infection.

Clear differences in the class mean spectra were observed primarily in the upper fingerprint (UF) and carbohydrate regions (Figure 8). This was consistent with specific spectral biomarkers extracted via multivariate analysis by way of cluster analysis and through PCA loadings (Figure 9). This revealed that the majority of variance within the spectral data of the MIR fingerprint were due to plant infection. The main biochemical variations and their potential compound identity were tentatively assigned and discussed. The most predominant modifications were detected in the spectral UF and carbohydrate regions, which were consistent with changes occurring in plant leaves because of colonization and attack by *B. cinerea* including tissue degradation and necrosis (Asselbergh et al. 2007; El Oirdi et al. 2011).

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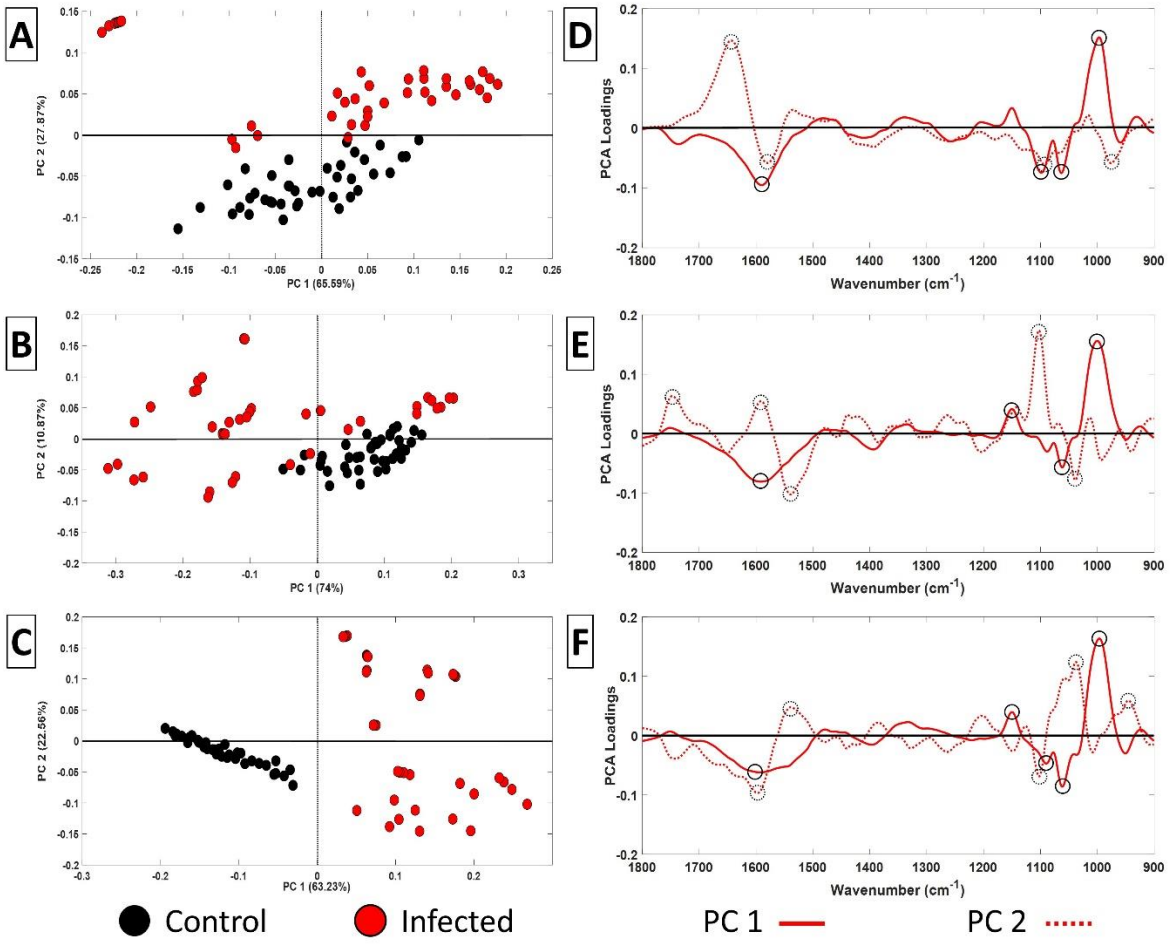

Figure 9: PCA 2-dimensional scores plots (A-C) and corresponding loadings (D-F) of pairwise control (black) versus *B. cinerea* infected (red) tomato leaf spectra at 48 (A and D), 96 (B and E), and 144 (C and F) hours Post Infection

Supervised PCA-LDA analysis completely segregated infected from non-infected plants at PS, IS, and AS stages of plant disease, showing promisingly high classification accuracy for applied disease detection in the field (Figure 10). This was confirmed with a minimum accuracy of 99% proving that this approach is highly suitable for pre- and post-symptomatic disease detection (Table 3). Because plant tissue and pathogen were intertwined in this experiment, this approach was categorized as direct pathogen detection (Sankaran et al. 2010).

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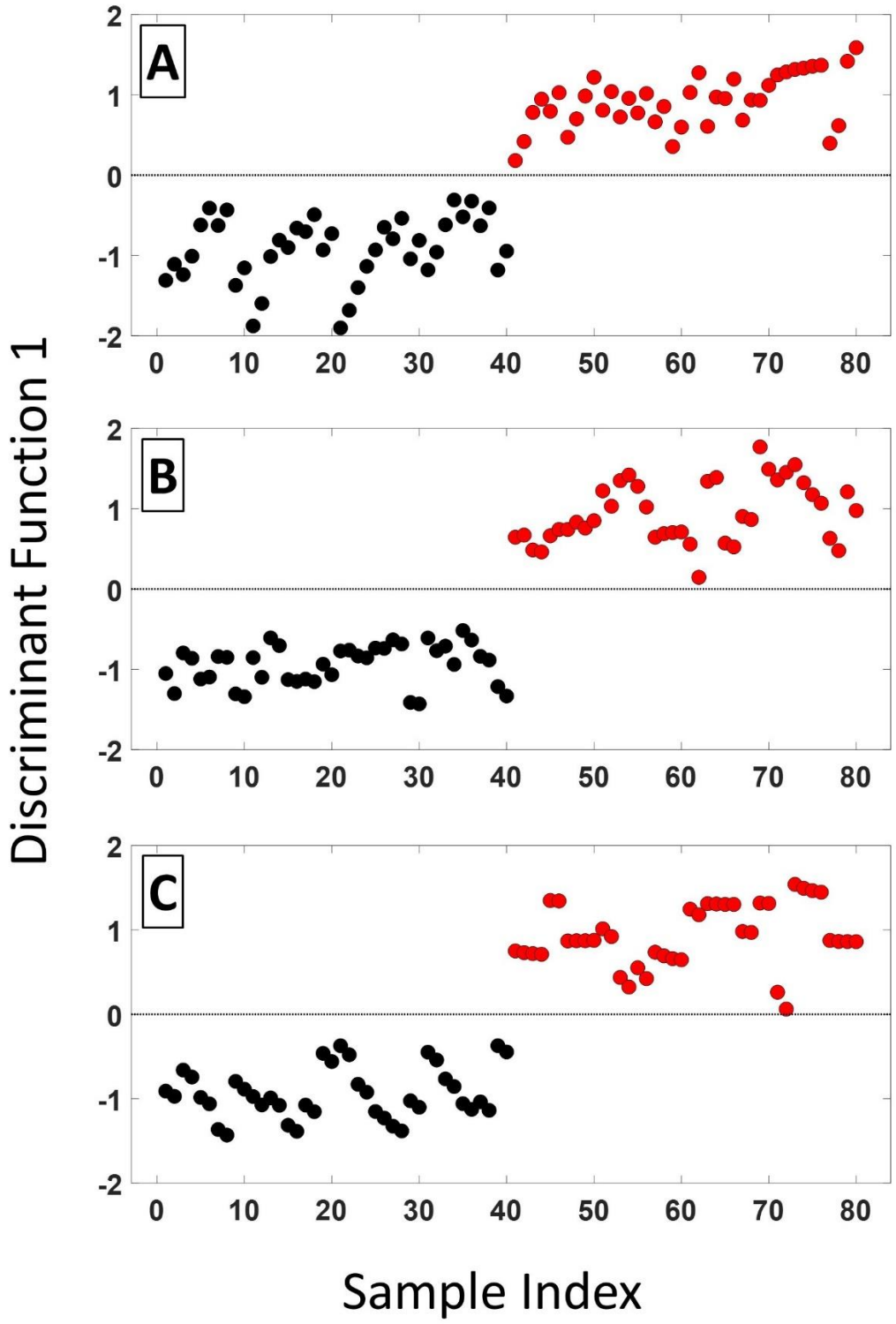

Figure 4: PCA-LDA scores plots of pairwise control (black) versus *B. cinerea* infected (red) tomato leaf spectra at 48 (A), 96 (B), and 144 (C) hours Post Infection

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Table 3: Classification results of control versus infected tomato plants using PCA-LDA

Infection Category	Accuracy	Sensitivity	Specificity
Pre-symptomatic	100%	100%	100%
Intermediate Symptomatic	99%	100%	97%
Advanced Symptomatic	100%	100%	100%

Rapid sensor-based disease detection will help reduce crop loss and increase food security overall, by facilitating the optimization of crop protection products, limit their overuse, while also reducing human and environmental exposure to harmful chemicals. Although promising among sensor technologies, further research is required in the field of MIR biospectroscopy based disease detection. Specifically, increased studies on intact plants *in vivo* with a focus on model plants/crops, as well as the evaluation of portable equipment suitable for the field (Skolik et al. 2018). Additionally, slight re-tooling of currently available MIR spectroscopy equipment, will permit further proof-of-concept field trials to be instigated in the near future, as has recently been achieved through the use of portable and handheld Raman spectrometers (Egging et al. 2018; Faber and Kurouski 2018; Yeturu et al. 2016). An unexplored aspect of MIR biospectroscopy is the use of acquisition modes for liquid and gaseous samples, which to date remain virtually unexplored, but offer additional potential for disease detection and plant-environment interactions relevant to crop biology. While the spectrochemical analysis of intact plant-pathogen systems is still in the beginning stages, the rapid growth of this field and the largely untapped potential of this technology will ensure its future contribution to the fields of plant and crop science

Discussion

Experimental data obtained throughout this project indicates that vibrational spectroscopy methods, especially ATR-FTIR spectroscopy can generate high quality spectra of intact plant leaves and fruit for disease detection purposes. Using various data analysis models, differences in healthy and diseased plants including fruit can be detected and characterized. Because there is only a limited amount of work done on intact plants, plant tissues, and whole fruit, it is difficult to compare with published literature and therefore presents a novel approach to the development of MIR biospectroscopy as a sensor technology for horticulture. In so far, it will take more time before the mechanisms, which are being measured and which are responsible for successful disease detection are clearly identified and incorporated into the plant sciences. Nonetheless, simple classification of treatments using classifier algorithms

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has been very successful providing a basis for commercial sensor development using MIR biospectroscopy methods. The high success rate of classifier algorithms for autonomous sorting of fruit and identification of abnormal states in plants, suggest that sensor development may be facilitated without knowing the mechanistic changes taking place. Throughout the research, several trends become clear including the ability to adapt biospectroscopy to various intact plant systems and that the performance of portable or semi-portable spectrometers is sufficient for horticultural applications. The difficulty of getting whole plants into contact with currently available biospectroscopy equipment, makes the evaluation of these methods for field applications challenging, except for portable Raman systems, which have recently shown promise to this end (Egging et al. 2018; Faber and Kurouski et al. 2018; Yeturu et al. 2016). In any case, identifying infection category of diseased plants, stress phenomena, as well as shelf life, ageing, or otherwise compromised plants and fruit can be achieved in most cases and on a variety of species. For these applications, ATR-FTIR, especially in macro mode, appears to be more appropriate for whole plant analysis compared to Raman spectroscopy due to the higher variability within Raman spectra attributed to the different penetration depth and interrogation area of these methods. Raman spectra, due to the small laser spot size and deeper sample penetration, generally shows higher variability as it measures more tissue layers (Butler et al. 2016). In contrast ATR-FTIR interrogates shallow surface structures and penetrates only slightly into the sample resulting in more reproducible and 'robust' spectra. However, MIR and Raman spectroscopy are complementary and work best when used in combination.

In combination with previous publications, the work here further supports the practicality of biospectroscopy, and extends its use as a physiological sensor capable of detecting stress-like responses. This demonstrates albeit preliminarily, the potential for vibrational spectroscopy to become an agricultural sensor technology for routine use. Given the complex nature of plants grown in agricultural settings, especially outdoors, MIR biospectroscopy will likely become one of many sensors within a multi-sensor array. As part of a multi-sensor assembly, biospectroscopy methods will fall into the category of proximal sensors requiring immediate proximity to the sample being analysed. It should be noted that hand held sensors reliable enough to augment/replace expert growers/pathologists for large scale routine screening would be most useful, especially for the pre-symptomatic detection of disease to maximize the effectiveness crop protection products.

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Conclusions

- Experimental data obtained indicates that vibrational spectroscopy can generate high quality spectra of intact plant leaves and fruit, demonstrating greenhouse/field applicability.
- Multivariate analysis provides a novel angle on the biochemical changes and biology related to the observed changes in spectra caused by healthy growth and development or by disease.
- Classifier algorithms applied to spectral data are have shown a high degree of accuracy suggesting rapid adaptations to industry are potentially beneficial.
- More data analysis is needed to completely evaluate the full potential of these sensors for commercial applications.
- Results from this PhD research suggests that MIR biospectral sensors will be effective for the pre-symptomatic detection of plant disease, where the efficacy of crop protection products may be optimized.
- Virtually any crop and associated pest can benefit from MIR spectral analysis for the purposes of disease detection or health monitoring.
- Biospectroscopy remains an exceptionally strong candidate sensor technology for further development and incorporation into multi-sensor platforms aimed at precision horticultural systems.
- MIR biospectroscopy may be incorporated into the experimental phase of integrated pest management.
- Once adapted for commercial use, biospectroscopy has the potential to significantly reduce crop loss.

Knowledge and Technology Transfer

Knowledge transfer was received through discussions with spectroscopists in the biomedical field, where specifically data analysis approaches were discussed (2015-2018). Technology transfer was provided by contributing oral presentations in Nottingham (2016), Lancaster Environment Centre (2015-2018), and Lancaster Science and Technology Conference (2017). Outreach work was provided as a PhD tutor through the Brilliant Club, teaching biospectroscopy at various schools at the Key Stage 4 and 5 level.

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Glossary (Abbreviations)

AS	Advanced Symptomatic
ATR	Attenuated Total Reflectance
DS	Developmental Stage
FTIR	Fourier Transform Infrared
IS	Intermediate Symptomatic
PCA	Principal Component Analysis
LDA	Linear Discriminant Analysis
MIR	Mid-infrared
PS	Pre-symptomatic
RS	Ripening Stage
SVM	Support Vector Machine

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Appendices

Supplementary Information

Determination of Developmental and Ripening Stages of Whole Tomato Fruit using Infrared Spectroscopy and Chemometrics

Table S1: Development stages of tomato fruit *S. lycopersicum* (cv. Moneymaker), corresponding spectral classes, and their AMS (USDA) grade designation (Kader and Morris 1976; Sargent and VanSickle 1996; Maul et al. 1998)

Developmental Stage (dpa)	Spectral Class	Average Weight (grams)	Average Diameter (cm)	AMS (USDA) Classification
04	DS01	2.8±0.2	0.62±0.02	M-1
08	DS02	4.9±0.1	1.16±0.03	M-1
12	DS03	6.8±0.1	1.96±0.07	M-2
16	DS04	12.8±0.2	2.99±0.04	M-2
20	DS05	21.5±0.3	4.84±0.09	M-2/M-3
24	DS06	51.6±0.2	5.39±0.08	M-3 (small)
28	DS07	88.2±0.5	6.41±0.13	M-3 (medium)
32	DS08	145.7±1.0	7.01±0.07	M-3 (large)
36	DS09	176.8±2.3	7.71±0.05	M-4 (extra-large)

Table S2: Ripening stages of tomato fruit *S. lycopersicum* (cv. Moneymaker), corresponding AMS (USDA) ripening and spectral class designation (Sargent and VanSickle 1996; Maul et al. 1998). Fruit used for ripening stages had an average diameter of 7.31±0.24cm.

Ripening Stage	Spectral Class	AMS/USDA Description
Mature Green	RS01	Fruit surface is completely green; shade may vary light to dark
Breaker	RS02	Break in colour from green to tannish-yellow, pink, or red on not more than 10% of the surface colour
Turning	RS03	10%-30% of the surface is not green; the aggregate shows a definite change from green to tannish-yellow and/or pink/red colour
Pink	RS04	30%-60% of the surface is not green; the aggregate, shows pink or red colour
Light Red	RS05	60%-90% of the surface is not green; the aggregate shows pinkish-red or red colour
Red (Ripe)	RS06	> 90% of the surface is not green; aggregate shows red colour

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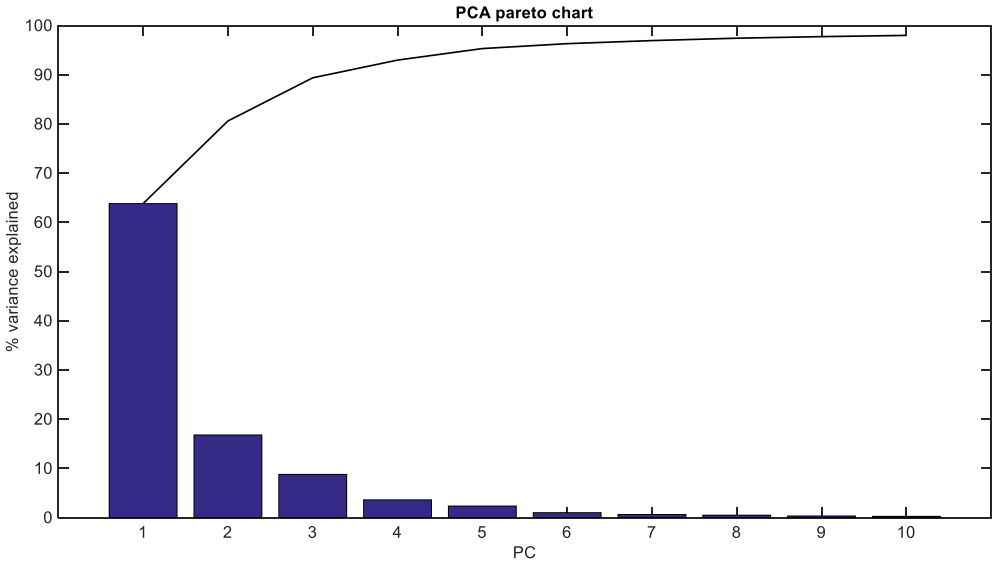

B

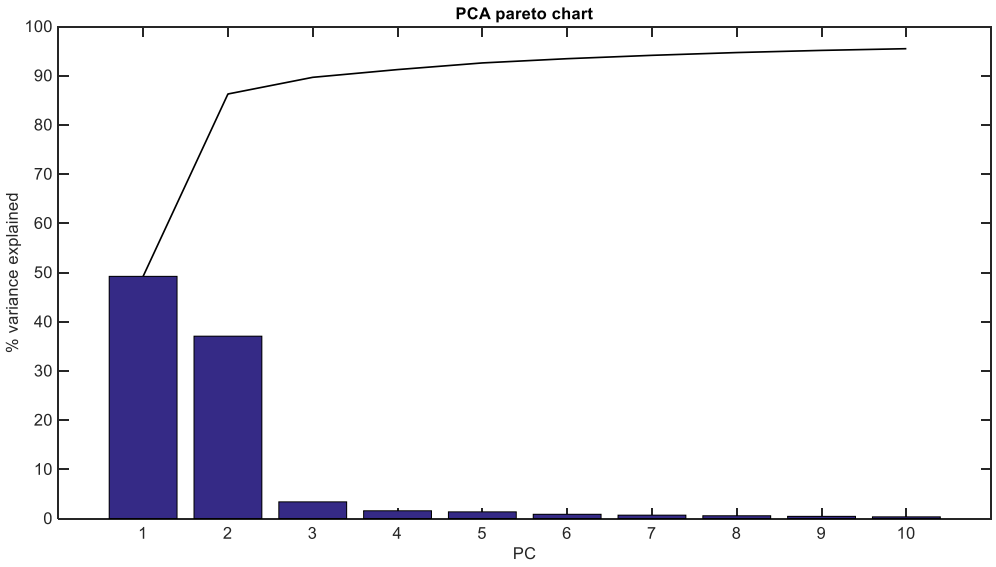

Figure S1: PCA Optimization for development (A) and ripening (B) groups using the Pareto function showing capture of >99% variance in the first 10 PCs.

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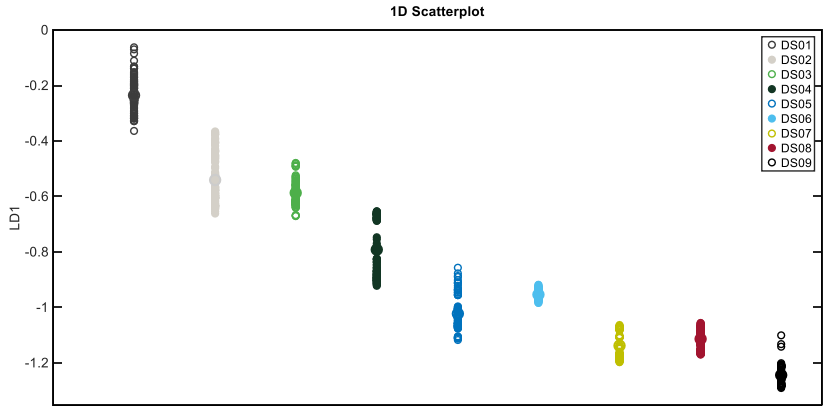
A



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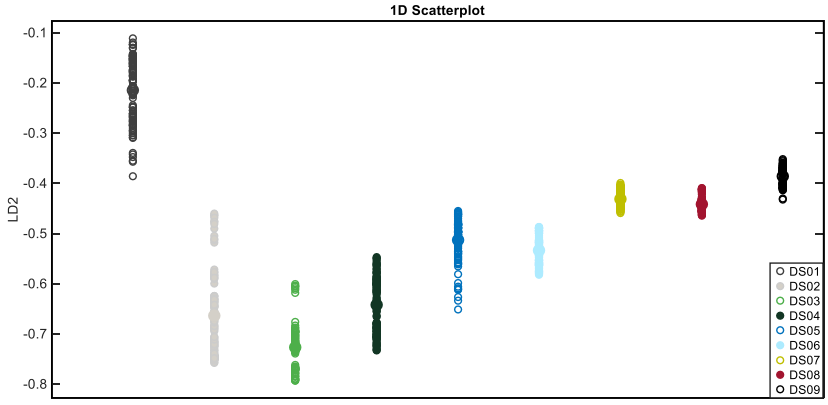

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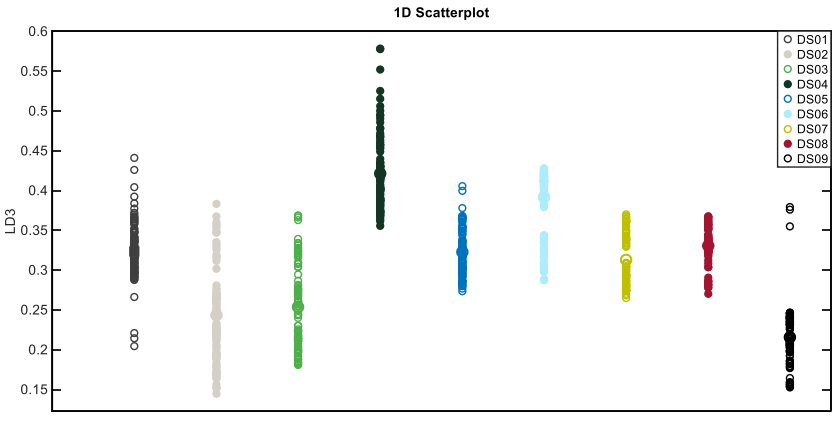

Figure S2: PCA-LDA scores plot for development groups showing LD1 (A), LD2 (B), and LD3 (C).

A

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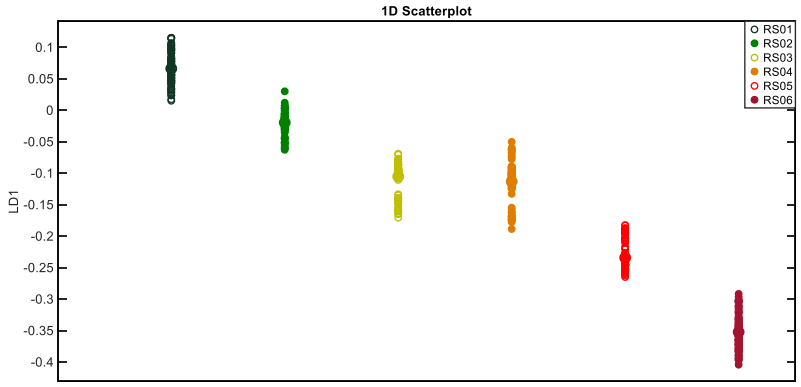

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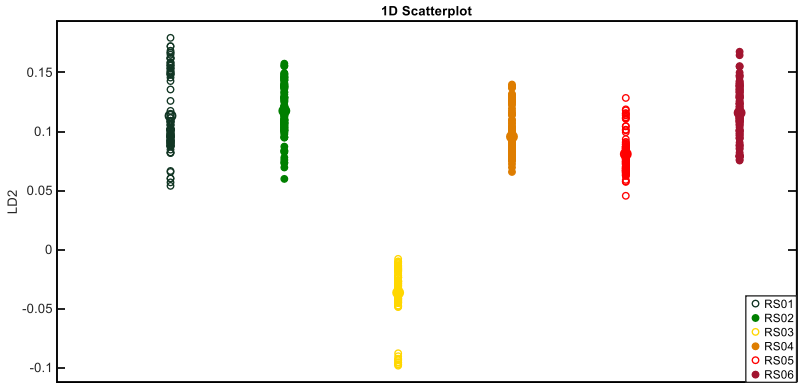

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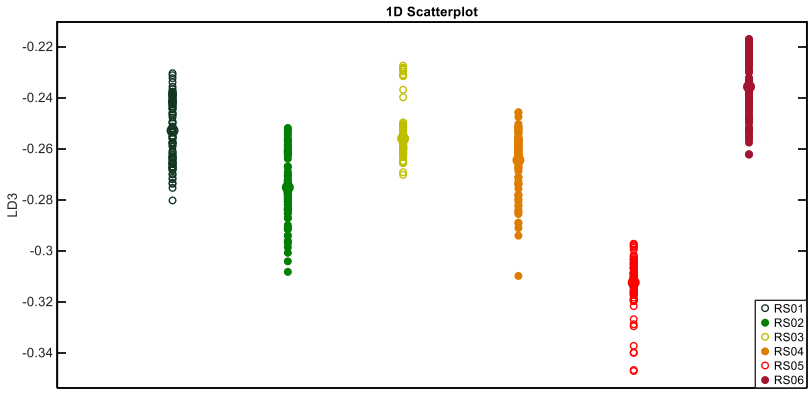

Figure S3: PCA-LDA scores plot for ripening groups showing LD1 (A), LD2 (B), and LD3 (C).

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Tomato Development

SVM Results

Confusion matrix

	DS01	DS02	DS03	DS04	DS05	DS06	DS07	DS08	DS09
DS01	100%	0%	0%	0%	0%	0%	0%	0%	0%
DS02	0%	100%	0%	0%	0%	0%	0%	0%	0%
DS03	0%	0%	100%	0%	0%	0%	0%	0%	0%
DS04	0%	0%	0%	100%	0%	0%	0%	0%	0%
DS05	0%	0%	0%	0%	100%	0%	0%	0%	0%
DS06	0%	0%	0%	0%	0%	100%	0%	0%	0%
DS07	0%	0%	0%	0%	0%	0%	100%	0%	0%
DS08	0%	0%	0%	0%	0%	0%	0%	99%	1%
DS09	0%	0%	0%	0%	0%	0%	0%	0%	100%

Cross-validation figures of merit

	Sensitivity	Specificity
DS01	100%	100%
DS02	100%	100%
DS03	100%	100%
DS04	100%	100%
DS05	100%	100%
DS06	100%	100%
DS07	100%	100%
DS08	100%	99%
DS09	99%	100%

Figure S4: SVM confusion matrix and class cross-validation results of spectral classes for development.

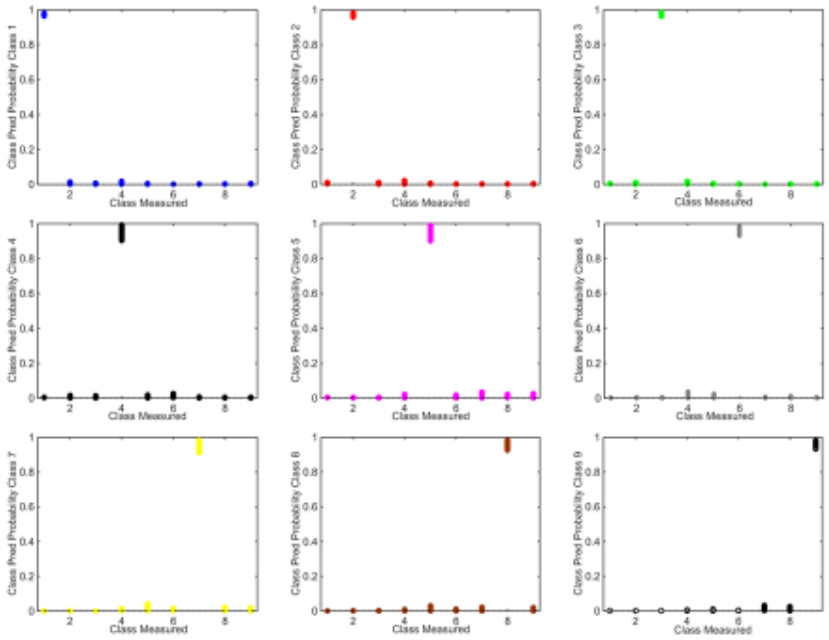

Figure S5: Class predictive probability results for individual spectral classes for development (in order DS01-DS09).

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Tomato Ripening Stages

SVM Results

Confusion matrix

	RS01	RS02	RS03	RS04	RS05	RS06
RS01	100%	0%	0%	0%	0%	0%
RS02	0%	100%	0%	0%	0%	0%
RS03	0%	0%	99%	1%	0%	0%
RS04	0%	0%	0%	100%	0%	0%
RS05	0%	0%	0%	0%	100%	0%
RS06	0%	0%	0%	0%	0%	100%

Cross-validation figures of merit

	Sensitivity	Specificity
RS01	100%	100%
RS02	100%	100%
RS03	100%	99%
RS04	99%	100%
RS05	100%	100%
RS06	100%	100%

Figure S6: SVM confusion matrix and class cross-validation results of spectral classes for ripening.

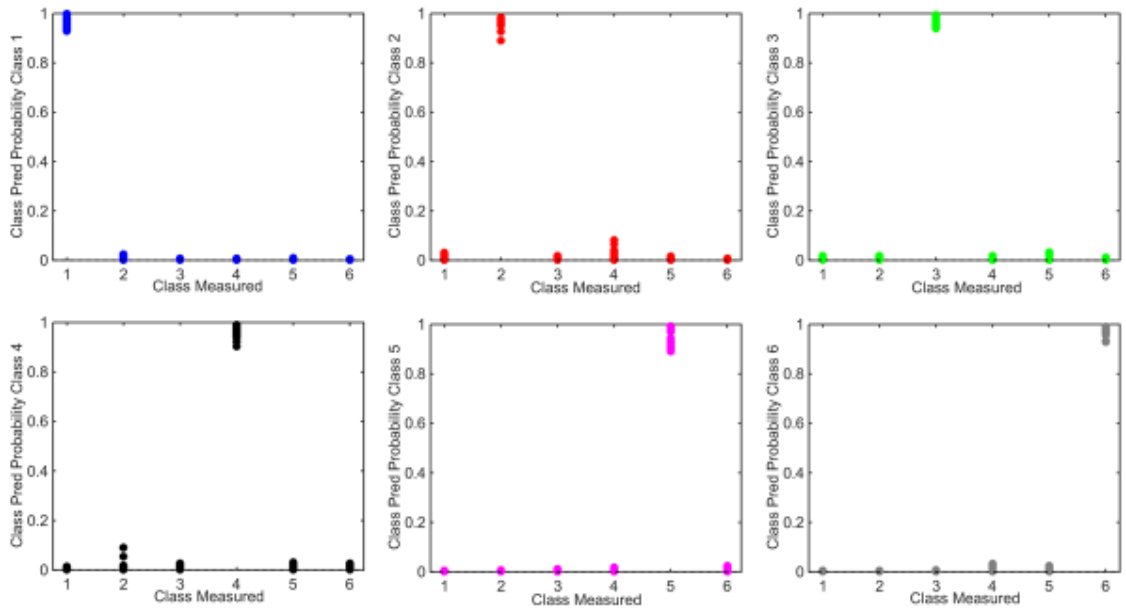

Figure S7: Class predictive probability results for individual spectral classes for ripening (in order RS01-RS06).

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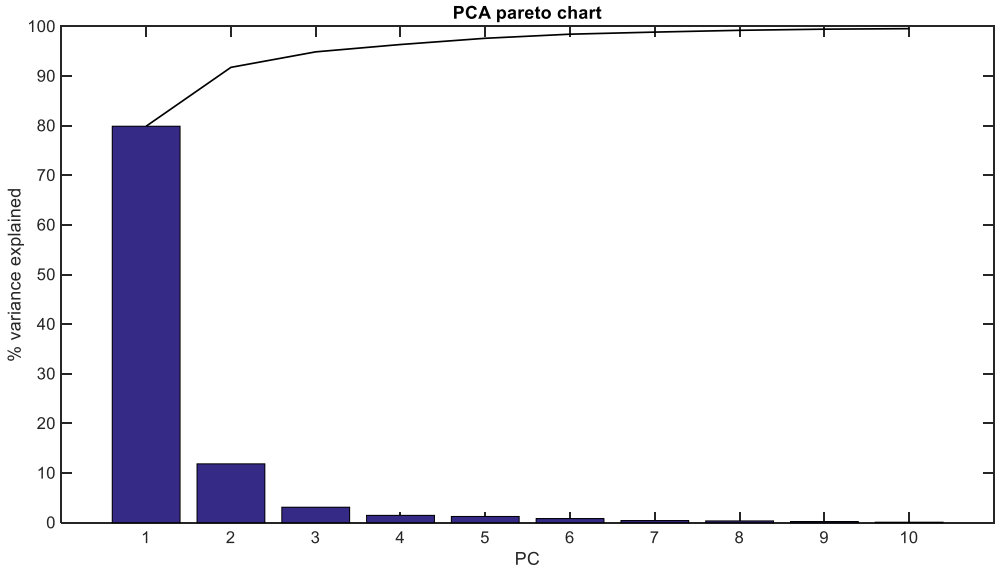

Figure S8: PCA optimization using the Matlab *pareto*-function

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Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy-Coupled Chemometrics Directly Detects Pre- and Post-Symptomatic Changes in Tomato Plants Infected with Botrytis cinerea

Table S3: *B. cinerea* infected tomato and observed symptomatic used for ATR-FTIR analysis (corresponding to Figure 1)

Infection Category	**Description**
Mock Control Plants (48, 96, and 144 h)	*Asymptomatic:* No visual symptoms were observed at any time during the study. Plants were observed for an additional 5 d to ensure no development of symptoms post-analysis.
Infected Plants 48 h	*Pre-symptomatic:* No visual symptoms observed: plants as described for mock controls. Plants measured at 48 h post-infection were observed for an additional 5 days to ensure pathogen colonization and symptom development as described for 96 and 144 h post-infection.
Infected Plants 96 h	*Intermediate symptomatic*: Early stages of infection visually apparent: vein and tissue discoloration (yellowing), isolated small variable lesions covering <50% of leaf area, slight leaf curling observed.
Infected Plants 144 h	*Advanced symptomatic:* Late stages of infection: large lesions covering >50% of leaf area, necrotic and or desiccated tissue, visible hyphae, pathogen sporulation.

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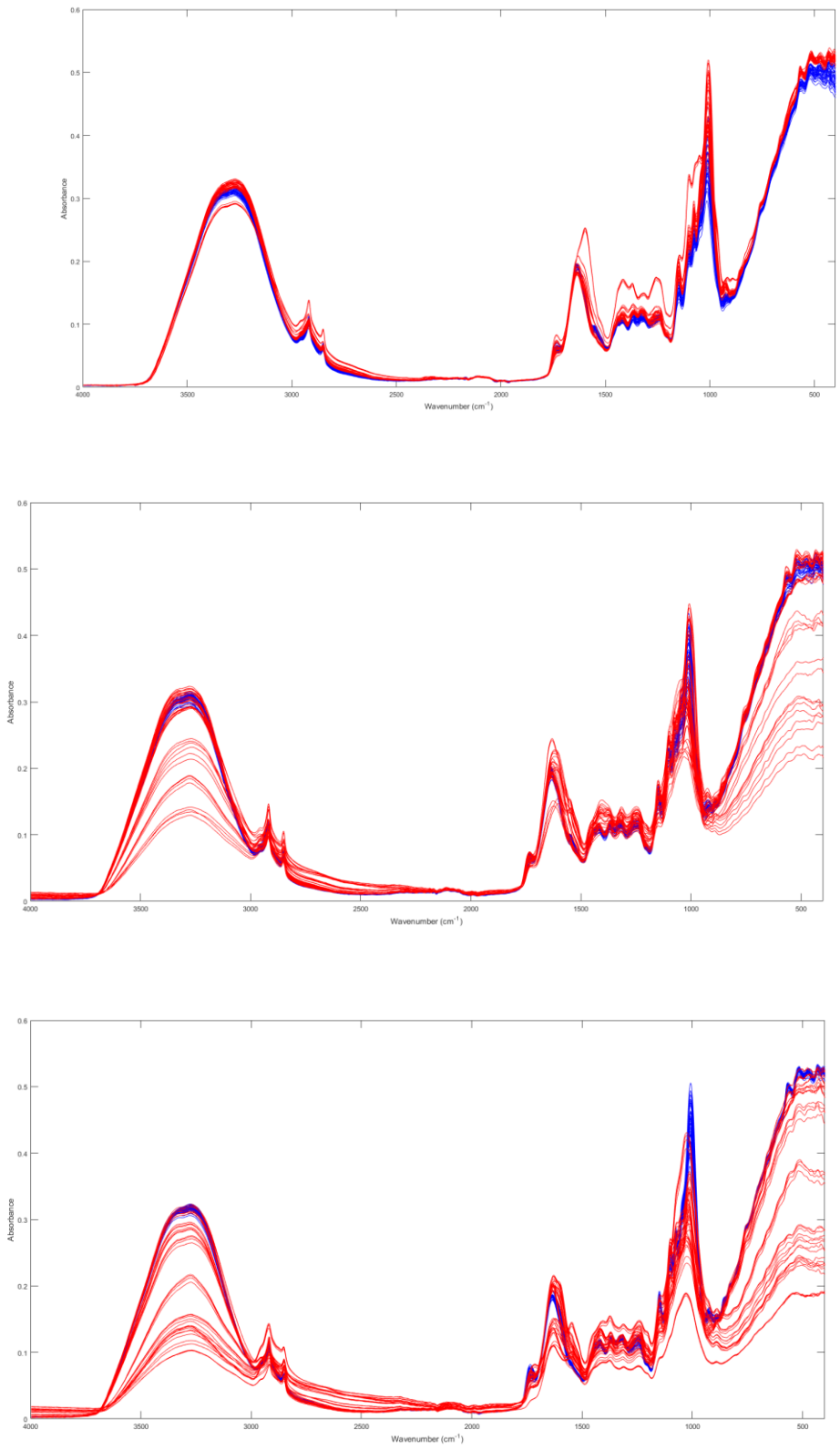

Figure S9: Raw spectral data for pairwise comparisons at 48 (PS), 96 (IS), and 144 (AS) h Post Infection (blue: controls; red: infected)

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SI Table 2: Confusion matrices for cross-validated PCA-LDA

Time (hours)		**Control**	**Infected**
48 h (PS)	Control	100%	0%
	Infected	0%	100%
96 h (IS)	Control	100	0
	Infected	3	97
144 h (AS)	Control	100	0
	Infected	0	100

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