Project title:	Sensor based pre-symptomatic detection of pests and pathogens for precision scheduling of crop protection products
Project number:	CP119
Project leaders:	Dr Martin McAinsh, Lancaster University Prof Frank Martin, University of Central Lancashire
Report:	Annual Report, 2017
Previous report:	Annual Report, 2016
Key staff:	
Location of project:	Lancaster Environment Centre, Lancaster University
Industry Representative:	
Date project commenced:	01.11.2015
Date project completed (or expected completion date):	01.11.2018

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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.]

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.

Dr Martin McAinsh

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CONTENTS

GROWER SUMMARY

Headline

There is a potential application for the development of proximal sensor devices for the detection of abnormalities in plants and crops that are associated with pest and pathogen effects. At this stage however it is difficult to quantify the financial benefits of the evaluated sensors especially with respect to their current cost of operation. As such no change to growing practice is currently advised.

Background

This project is intended to evaluate the feasibility of developing biospectroscopy (MIR and Raman spectroscopy) as sensor technologies in various horticultural settings to mitigate crop loss. Rapid non-destructive sensors, such as biospectroscopy, will aid in the development of sustainable technologies for the reduction of crop loss to pests and pathogens pre and post-harvest, thereby improving the rational use of crop control measures and reducing negative environmental impacts.

Summary

At this point and due to exploratory nature of the project, there is insufficient material to expand this section reliably, especially because data analysis for many of the results is on-going and need to be validated through reproducibility.

Financial Benefits

Due to the stage of development of the technology, prospective financial benefits cannot be meaningfully analysed.

Action Points

- Currently no change to grower practice is advised

SCIENCE SECTION

Introduction

Food security and production sustainability is projected to become more challenging as global population rises. Additionally, horticultural losses in the form of pre- and post-harvest crop losses caused by adverse environmental conditions, pests, and pathogens impact negatively on food security, causing pre-consumer crop losses of as much as 40% (Gerland et al 2014). This contributes significantly to problems including economic damage, malnourishment, natural resource depletion, and can lead to excessive use of crop protection products (Savary et al. 2012). To avoid such impacts on crop production (Ray et al 2013), solutions must be found to reduce of crop loss in pre- and post-harvest settings (crop cultivation, harvest, packaging, distribution, consumer waste, etc.). The numbers of variables involved in crop cultivation, specifically with regard to mitigating loss are impressive as well as challenging and this makes development of methods for crop monitoring difficult. This has left tasks such as plant disease detection and identification, to expert growers who currently have access to a variety of technologies many of which are destructive, laborious, difficult to use, and not necessarily representative of often heterogeneous field settings (Mahlein et al. 2016).

Technologies enabling non-destructive detection of the negative effects of biotic and abiotic stressors on plants and harvested products can therefore contribute to sustainable agricultural production. Candidate technologies for helping reduce crop losses include non-destructive optical sensors, but the number of variables and complexity of modern farming suggests that a range of technologies will be needed (Sankaran et al 2010; 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. From a practical perspective, these sensor technologies must be adaptable from crop to crop, and usable in variable field conditions. Providing growers with tools to detect, identify, and even quantify disease, damage or other parameters relevant to quality assurance is a high priority for development.

Vibrational spectroscopy in biology (biospectroscopy) has been successful as a biomolecular sensor for disease detection and discrimination of abnormal cells and tissues based on spectral alterations. Biospectroscopy has become an enormous 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 physiological sensor in plant science for horticultural applications. Vibrational methods, including Mid-Infrared (MIR) and Raman spectroscopy are

among the most well-known techniques 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 within a sample (Kazarian and Chan 2013). Light energies within the MIR range correspond to vibrational and rotational modes of biochemical functional groups present in proteins, lipids, carbohydrates, and nucleic acids (Baker et al. 2014). Disparate light-matter interactions of IR 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).

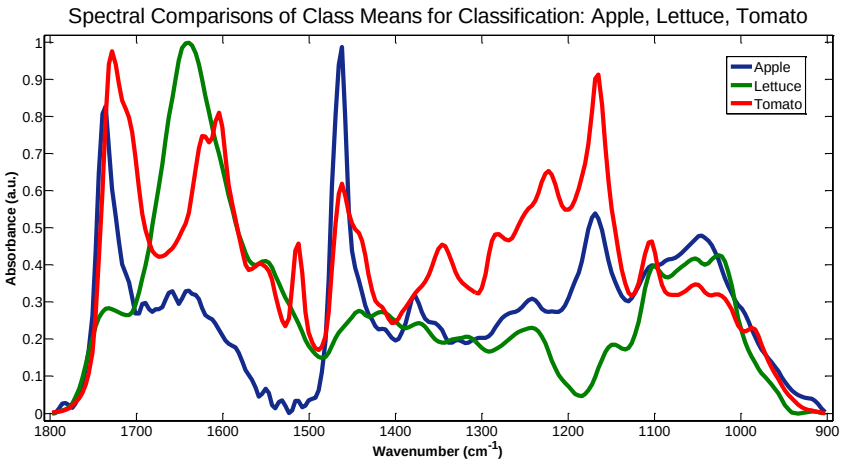

Figure 1: Attenuated total reflectance Fourier transform infrared (ATR-FTIR) class mean spectra of apple peel (blue), lettuce leaves (green), and tomato skin (red) over the 'fingerprint' region (1800-900cm^{-1}) of the MIR range.

Modern MIR spectroscopy generates highly characteristic spectral 'fingerprints' or 'signatures' which represent a collection of hundreds to hundreds of thousands of potential bonds (Butler et al. 2016). These can enable detection of useful disease markers or biomarkers associated with an abnormal state (damage, disease, contamination, deterioration, etc.). 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 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 may 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 be used to supplement expert evaluations when

needed, automatic diagnostic frameworks, even in the absence of experts, will be a necessity as part of sustainable practices.

This PhD project is aimed at evaluating biospectroscopy as a potential solution, through the pre-symptomatic detection of pests and pathogens to reduce crop loss. With future investigations and collaboration with industry partners, major problem areas will be identified at the pre- and post-harvest stages of the food supply chain, where biospectroscopy may be employed.

Materials and methods

Plant Growth Conditions

Materials and methods for this project differ broadly with regard to 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 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.

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. With the exception of very few experiments, all samples (plants and fruit) were analysed intact. Typically de-ionized water and a clean cloth was used to remove dust, dirt, and obvious debris from fruit prior to spectral analysis.

Several experiments made use of pathogen infection protocols using *Botrytis cinerea*. Infection protocols used were previously described by Audenaert et al (1999).

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 project.

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. New in year 2 of this 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 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 variable 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

Building on previous results from year 1, the year 2 experimental design was focused to study tomato as the primary plant/crop model. Tomato (*Solanum lycopersicum*) was previously studied with ATR-FTIR and Raman spectroscopy and was chosen in conjunction with microscopic pathogens such as *Botrytis cinerea* to characterize spectral alterations in response to tissue necrosis associated with this pathogen. Several experiments were conducted in both whole plants and tissue samples (leaflets specifically). Multiple sensors were combined in whole plant experiments to cover both physiological measurements using infrared gas analysis (IRGA) sensors such as LiCor photosynthesis sensors, combined with IR spectroscopy.

In addition to the focused experimental design of year 2, several datasets were re-visited to evaluate classifier performance for automated detection/decision based on IR spectroscopy data. Details are given in the individual experiments presented below.

EXPERIMENT: Investigating *Botrytis cinerea* Infection in Tomato Plant Tissue using ATR-FTIR Spectroscopy and Multivariate Analysis

Tomato leaf tissues (leaflets) were sprayed with solution inoculated with *B. cinerea* spores in order to compare healthy tissue with infected tissue based on their MIR spectra. Pictures (Figure R1.1) clearly show the deterioration of tomato leaves infected with the fungus. Control leaves showed no visual signs of decay or infection over the one week time course.

Figure R1.1: Progression of *Botrytis cinerea* infection in tomato leaf tissue (left to right at Day 1, 3,5, and 7)

ATR-FTIR spectra were obtained over the time course for both healthy and diseased tissue in order to compare the spectral alterations associated with each treatement (i.e. regular ageing/senescence versus infection). Second order differentiated and vector normalized pre-processed mean spectra for all groups in the study are shown in Figure R1.2.

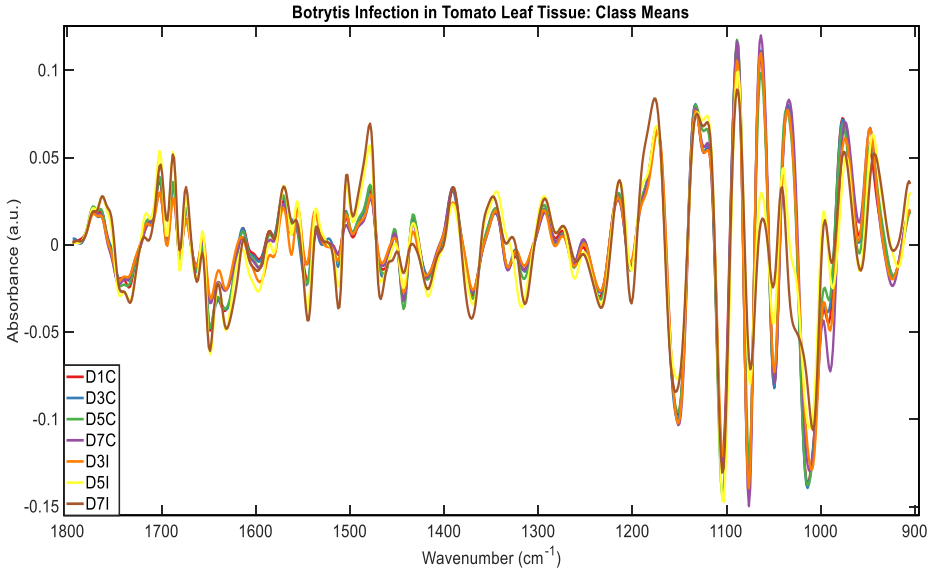

Figure R1.2: Class mean ATR-FTIR spectra of experimental groups (control and infected leaftlets at day 1, 3, 5, and 7)

Spectral alterations of both control leaflets and infected leaflets where categorized in reference to freshly removed leaf tissue from control plants on day 1 (Figure R1.3). While these have been tabulated, the analysis for this experiment is on-going. It was however determined that according to spectral data, there appears to be a potential mechanistic link between the natural progression of leaf senescence in control plants and the induced tissue decay of *B.cinerea* infected leaflets (figure not shown), although this has yet to be confirmed. What can be confirmed is that there is a statistical difference in LD1 scores between spectra from control and infected tomato plant tissue for all but day 7. On day 7, control leaflets showed similar spectral alterations to infected leaflets.

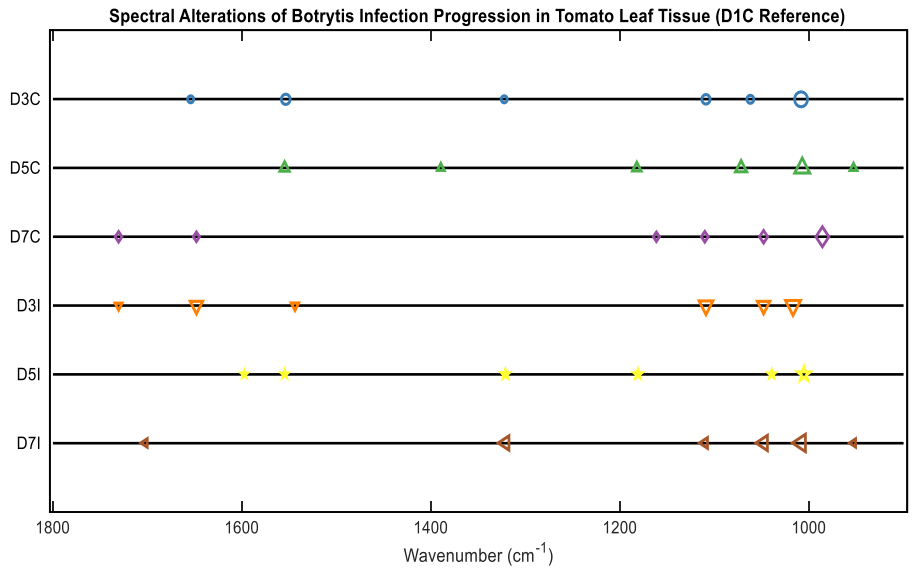

Figure R1.3: PCA-LDA cluster vector peak location plots showing the top 6 wavenumbers differing between experimental groups and fresh cut control leaflets on day 1

EXPERIMENT: Investigating *Botrytis cinerea* Infection in Whole Tomato Plants using Physiological IRGA and ATR-FTIR Spectroscopy: A Multi-Sensor Approach

Whole tomato plants were drop infected with solution inoculating spores of *B.Cinerea*. Leaves of intact plants were measured using a LiCor IRGA sensor measuring parameters of photosynthesis (Figure R2.1), transpiration, stomatal conductance, and internal carbon concentration. While no visual symptoms of the infection arose in infected plants, there appeared to be a physiological response on day 5 post infection (Figure R2.1). This response was consistent for the other parameters measured (transpiration, stomatal conductance, and internal carbon).

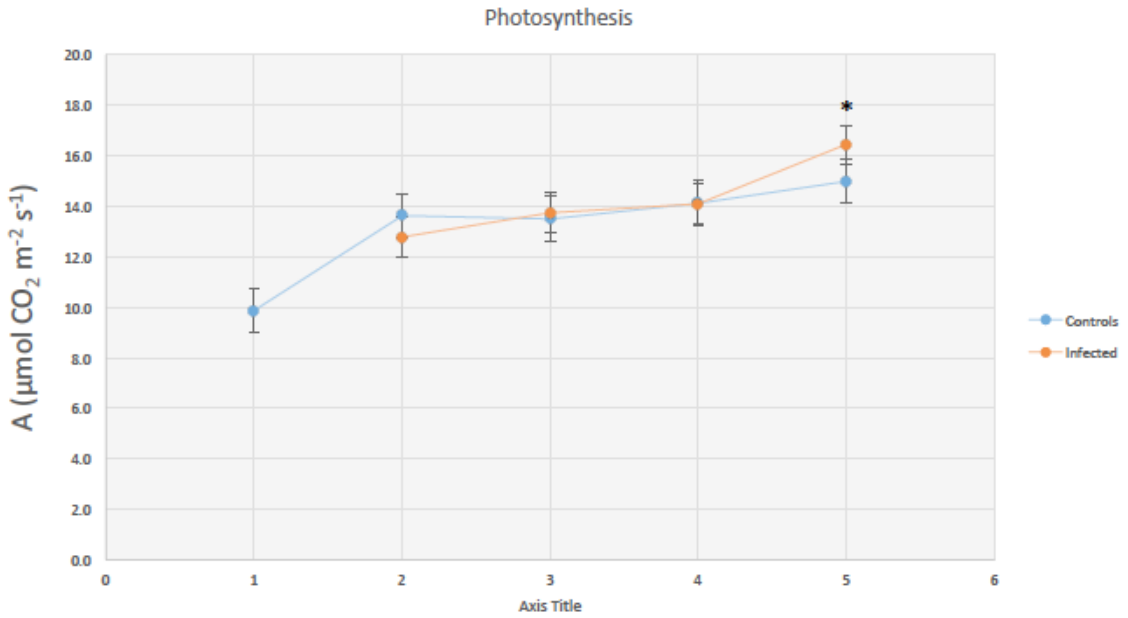

Figure R2.1: Physiological response to *B.Cinerea* infection in leaves of whole tomato plants

Comparing results obtained from IRGA measurements to ATR-FTIR spectroscopy showed a statistically significant response on day 2 and 3, as determined by ANOVA analysis of LD1 scores, which preceded physiological responses measured by the IRGA sensor (Figure R2.2; statistical indicators not yet added). This may suggest that ATR-FTIR spectroscopy may detect physiological changes (those measured) associated with pathogen infection prior to conventional IRGA sensors. However, it is difficult to confirm that the physiological changes arising on day 5, seen by IRGA measurements, as well as those detected by ATR-FTIR spectroscopy, are in fact due to the fungus and not another factor. Data analysis and exploration of the results for this experiment are still in progress and therefore serve only as preliminary results.

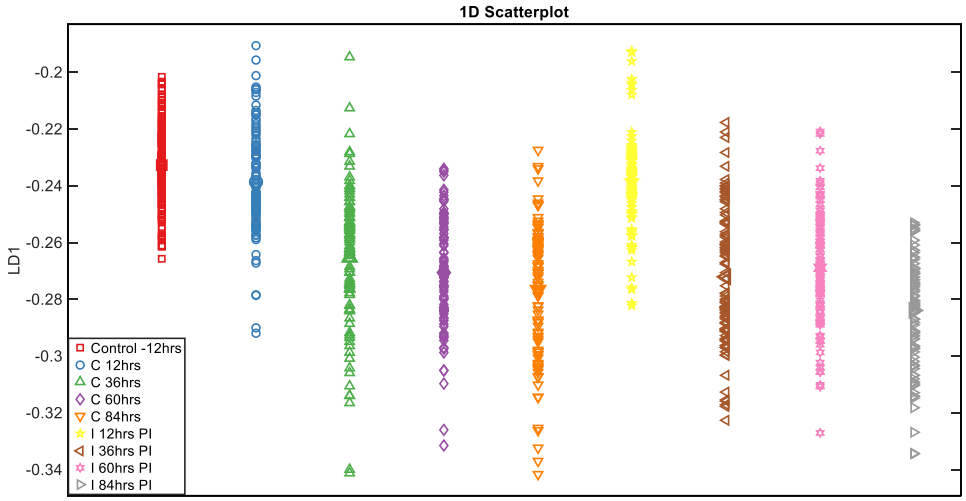

Figure R2.3: PCA-LDA scores of ATR-FTIR spectra obtained from whole tomato plant leaves for controls at day 1 (pre-infection), 2, 3, 4, and 5 and infected groups at day 2, 3, 4, and 5

EXPERIMENT: Characterizing Intact Tomato Fruit Development and Ripening using Semi Portable ATR-FTIR Spectroscopy

Due to the limited amount of research employing biospectroscopy in whole plants and fruit, it was necessary to characterize natural on-plant tomato fruit development and ripening to establish a baseline of spectral alterations in order to distinguish natural processes from abnormal conditions (damage, stress, infection, etc.). In an attempt to achieve this, greenhouse grown tomato fruits were measured intact at various stages of natural development and ripening. Figure R3.2 and R3.3 show clustering of developmental and ripening groups (On plant tomatoes shown in Figure R3.1).

Figure R3.1 On-plant tomatoes for development and ripening study

Development of tomato was distinguished at 9 separate stages ranging from very small immature through to mature green. ATR-FTIR spectra were obtained from 10 fruit (2 per plant) with 10 spectra taken from each fruit to increase the number of biological replicates and generate representative spectral clusters from each group for comparison. At each developmental stage there is overlap between stages suggesting the common spectral elements between groups and distinct features of each group. This is shown in figure R3.2.

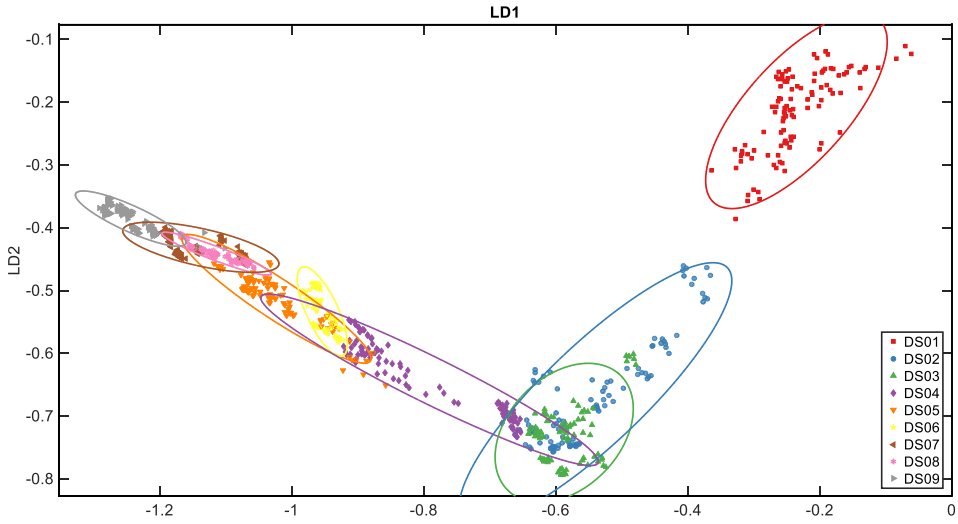

Figure R3.2: PCA-LDA scores of 9 distinct developmental stages of tomato

Similarly, ripening stages of tomato also formed distinct groups along both LD1 and LD2, with less overlap compared to developmental stages suggesting more pronounced differences at each stage of the ripening process (Figure R3.3). This may be due fewer distinguished groups during the ripening stages compared with development. All tomatoes analysed during the ripening process were of comparable size as to minimize data variance based on size, weight, etc. This is new work, which has yet to be fully analysed, specifically the spectral biomarkers responsible for data separation of development and ripening stages. However, preliminary results shown here suggest that ATR-FTIR spectroscopy accurately reflects the molecular level changes, which are also visible with the naked eye, associated with visible fruit growth and ripening. These results will be compared to store bought tomatoes of a different cultivar in order to determine both common biomarkers within tomato cultivars, if any, and the unique markers to each.

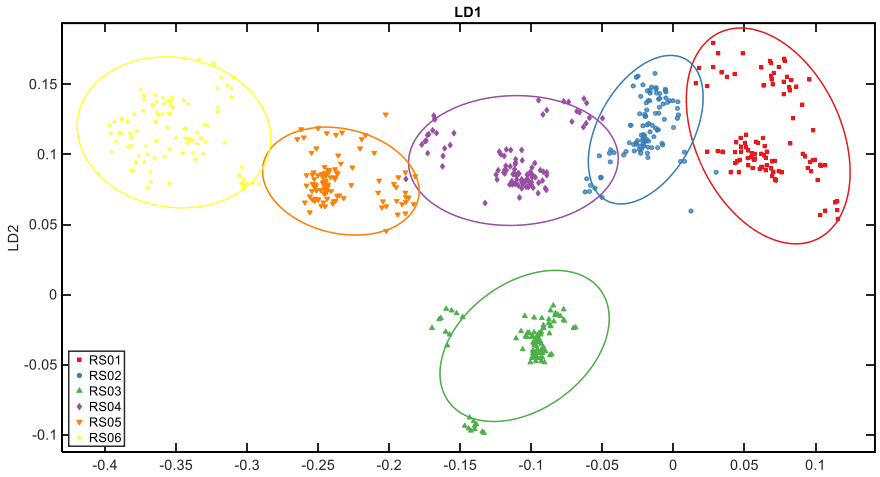

Figure R3.3: PCA-LDA scores of 6 distinct ripening stages of tomato

Preliminary classifier results for both fruit development (Figure R3.4) and ripening (Figure R3.5) stages showing very good accuracy for 'critical' classification, misclassification is likely due to the very similar molecular composition of fruit at these stages, such as development stage 5 (DS05) with a lower classification accuracy of approximately 50%. These stages, pending further analysis, may be classed as 'critical' or 'non-critical' depending on the industry application. In other words, if only a sub-set of the developmental stages need to be correctly identified, then these may be deemed 'critical', while all other stages may be deemed 'non-critical'.

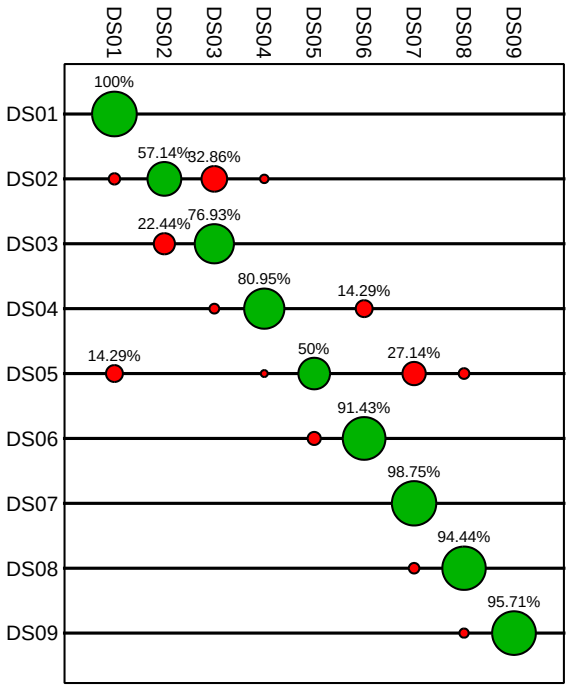

Figure R3.4: PCA-LDC classifier results for autonomous classification of developmental stages of tomato fruit

Classification accuracy for ripening stages (Figure R3.5) were exceptionally good with a minimum accuracy of 90.71%. From these results, it appears that ripening stages are more readily identified compared to developmental stages. These results will be adapted to visual ripening scales in order to develop methods for the objective determination of optimal ripeness, for example mature green, breaker, yellow, pink, light red, red, although other applications are possible depending on the exact needs of industry.

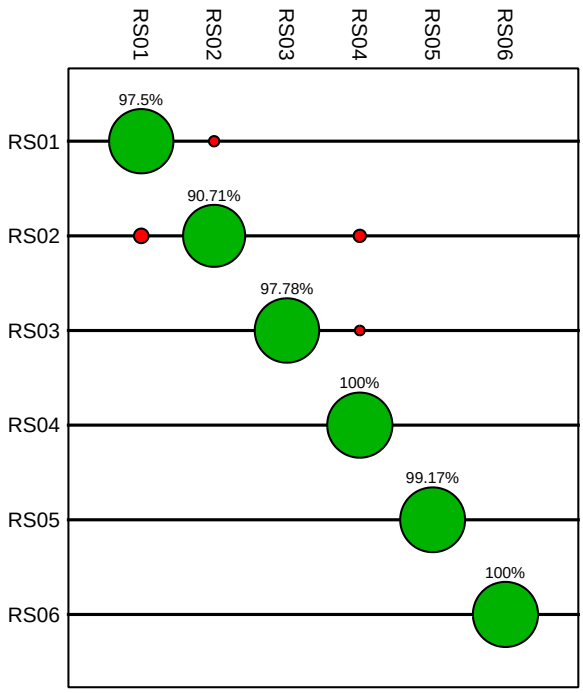

Figure R3.5: PCA-LDC classifier results for autonomous classification of ripening stages of tomato fruit

Discussion

Experimental data obtained to date indicates that vibrational spectroscopy methods, especially ATR-FTIR spectroscopy is capable of generating high quality spectra of intact plant leaves and fruit. 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 makes it difficult to compare with published literature and therefore presents a novel approach to the development of biospectroscopy as a sensor technology for horticulture. Although data analysis has not been finalized for most experiments, several trends become clear including the ability to adapt biospectroscopy to a number of intact plant systems. In so far, it will take

more time before the mechanisms, which are being measured and which are responsible for data separation in most cases, are clearly identified and incorporated into the plant sciences. However, simple classification of treatments using classifier algorithms has been very successful providing a basis for commercial sensor development using biospectroscopy techniques. This research shows the possibility to use this approach for whole plants and fruit. The difficulty of getting whole plants into contact with currently available biospectroscopy equipment, makes the evaluation of these methods for field applications difficult. In contrast, biospectroscopy may be more readily adapted for fruit analysis, especially post-harvest for high throughput screening. 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 with regard to the biomolecules measured. In any case, identifying infection category of diseased plants, stress phenomena, as well as shelf life, ageing, or damaged plants and fruit can be achieved in most cases and on a variety of produce (apple, lettuce, tomato, etc.). For these application, ATR-FTIR, especially in macro mode, appears to be more versatile and appropriate compared to Raman spectroscopy. The higher variability within Raman spectra is likely 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. In contrast ATR-FTIR interrogates small tissue sections and penetrates only shallow into the sample resulting in more reproducible and therefore robust spectra. However, these techniques are complementary and may be variably applied in different settings depending on specific research or application needs.

Spectral changes and the associated wavenumber markers are tentatively assigned as responsible for the observed alterations. Identified wavenumber markers will serve as starting points (input) for the development of a diagnostic framework. 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 monitoring of plants and their products with the potential for disease detection. Given the complex nature of plants grown in agricultural settings, especially outdoors, 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. What remains

elusive is the pre-symptomatic detection of spectral alterations prior to irreversible effects such as tissue necrosis and disease spread.

Future investigation will include extensive characterization of differences between healthy and mildly infected leaves, to determine potential spectral biomarkers associated specifically with pre-symptomatic development of tissue necrosis as a result of disease such as leaf spot. Additionally, the notion of conserved and specific stress responses will be explored. In summary, experimental applications of biospectroscopy in the form of ATR-FTIR and Raman spectroscopy are versatile and capable of measuring fresh plant materials including whole plants. Extensive changes in spectral clusters relating to sample category were observed in all experiments.

Conclusions

- Experimental data obtained to date indicates that vibrational spectroscopy is capable of generating high quality spectra of intact plant leaves and fruit.
- Analysis of completely unprepared samples maintains greenhouse/field applicability.
- Classifier algorithms applied to spectral data are have shown a high degree of accuracy suggesting rapid adaptations to industry.
- More data analysis is needed to completely evaluate the full potential of these sensors for commercial applications (aim for year 3).
- How successful these sensors will be for the pre-symptomatic phase is difficult to determine at this stage.
- Biospectroscopy remains an exceptionally strong candidate sensor technology for further development and incorporation into multi-sensor platforms aimed at horticultural detection systems.
- Once adapted for commercial use, biospectroscopy has the potential to significantly reduce crop loss.

Knowledge and Technology Transfer

To date the main form of technology transfer has been by way of conference contributions (posters, oral presentations, discussions). One publication is pending acceptance.

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Appendices

A comprehensive appendix is being compiled for inclusion in the final report as much of the experimental data analysis is still in progress and figures have not been formally composed.

