Sensor-Based Pre-Symptomatic Detection of Pests and Pathogens for Precision Scheduling of Plant Protection Products

A thesis submitted in partial fulfilment for the degree of Doctor of Philosophy

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Declaration

I, Paul Skolik, declare that the contents of this thesis are my own work. I have not submitted this work in any form for the award of higher degree at any other institution, other than Lancaster University. Chapters 2-4 have been peer reviewed and published due to this project. Chapter 5 has been submitted for publication.

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ABSTRACT

Providing global food security requires a better understanding of how plants function and how their products, including important crops are influenced by environmental factors. Prominent biological factors influencing food security are pests and pathogens of plants and crops. Traditional pest control, however, has involved chemicals that are harmful to the environment and human health, leading to a focus on sustainability and prevention with regards to modern crop protection. A variety of physical and chemical analytical tools is available to study the structure and function of plants at the whole-plant, organ, tissue, cellular, and biochemical levels, while acting as sensors for decision making in the applied crop sciences.

Vibrational spectroscopy, among them mid-infrared and Raman spectroscopy in biology, known as biospectroscopy are well-established label-free, nondestructive, and environmentally friendly analytical methods that generate a spectral "signature" of samples using mid-infrared radiation. The generated wavenumber spectrum containing hundreds of variables as unique as a biochemical "fingerprint", and represents biomolecules (proteins, lipids, carbohydrates, nucleic acids) within biological samples. Spectral "biomarkers" generated by biospectroscopy is useful for the discrimination of distinct as well as closely related biomaterials, for various applications. Applications within the plant and crop sciences has been limited to date, especially for the investigation of dynamic biological processes in intact plant tissues. Even more scarce is the application of biospectroscopy to plant interactions with pests and pathogens.

To adequately probe *in vivo* plant-environment interactions, surface structures of intact plant tissues such as leaves, and fruit need to be characterized. Infrared light energy can measure plant epidermal structures including the cuticle and cell wall for chemical profiling of different varieties and cultivars, as well as physiological applications such as plant health monitoring and disease detection. A review of the application of biospectroscopy to study plant and crop biology reveals the potential of biospectroscopy as a prominent technology for fundamental plant research and applied crop science. The application of biospectroscopy for *in vivo* plant analysis, to elucidate spectral alterations indicative of pest and pathogen effects, may therefore be highly beneficial to crop protection.

Highlighting the *in vivo* analysis capability and portability of modern biospectroscopy, ATR-FTIR provided an invaluable tool for a thorough spectrochemical investigation of intact tomato fruit during development and ripening. This contributes novel spectral biomarkers, distinct

for each development and ripening stage to indicate healthy development. Concurrently, this approach demonstrates the effectiveness of using spectral data for machine learning, indicated by classifier results, which may be applied to crop biology.

Complementary to monitoring healthy growth and development of plants and crops, is the detection of threats to plant products that compromise yield or quality. This includes physical damage and accelerated decay caused by pests and pathogens. Biochemical changes detected by ATR-FTIR using principal component analysis and linear discriminant analysis (PCA–LDA), for damage-induced pathogen infection of cherry tomato (cv. Piccolo), showed subtle biochemical changes distinguishing healthy tomato from damaged, early or late sour rot-infected tomato. Sour rot fungus *Geotrichum candidum* was detected *in vivo* and characterized based on spectral features distinct from tomato fruit providing biochemical insight and detection potential for intact plant–pathogen systems.

Pre-harvest detection of pests and pathogens in growing plants is paramount for crop protection and for effective use of crop protection products. Established previously as an exceptionally versatile bioanalytical sensor, for post-harvest applications, biospectroscopy was applied for the pre-harvest detection of microscopic pathogen *Botrytis cinerea* fungus infecting developing tomato plants. Compact MIR spectroscopy using ATR mode was adapted for the biochemical investigation of the plant-microbe interaction *S. lycopersicum* and *B. cinerea*, on the whole-plant level. Chemometric modeling including principal component analysis, and linear discriminant analysis were applied. Fingerprint spectra (1800-900 cm-1) were excellent discriminators of plant disease in pre-symptomatic as well as symptomatic plants. Spectral alterations in leaf tissue caused by infection are discussed. Potential for automatic decision-making is shown by high accuracy rates of 100% for detecting plant disease at various stages of progression. Similar accuracy rates using similar chemometric models are obtained for fruit development and ripening also.

Overall, this research showcases the biospectroscopy potential for development monitoring and ripening of fruit crops, damage and infection induced decay of fruit in horticultural systems post-harvest, complemented by pre-harvest detection of microscopic pathogens. Based on the results from experiments performed under semi-controlled conditions, biospectroscopy is ready for field applications directed at pest and pathogen detection for improved crop production through the mitigation of crop loss.

LIST OF ABBREVIATIONS (alphabetical)

ABA	Abscisic Acid
ANN	Artificial Neural Network
AS	Advanced Symptomatic
ATR	Attenuated Total Reflectance
AWLS	Automatic Weighted Least Squares
BaF_2	Barium Fluoride
CaF_2	Calcium Fluoride
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DS	Developmental Stage
DTGS	Deuterated Triglycine Sulphate
E	Energy (Photon)
ELISA	Enzyme-Linked Immuno-Sorbent Assay
FISH	Fluorescent *In-Situ* Hybridization
FPA	Focal Plane Array
FT	Fourier Transformation
FTIR	Fourier Transform Infrared
GA	Genetic Algorithms
GC	Gas Chromatography
GM	Genetically Modified
HCA	Hierarchical Cluster Analysis
HPLC	High Pressure Liquid Chromatography
HR	Hypersensitive Response
IPM	Integrated Pest Management
IR	Infrared
IRE	Internal Reflection Element
IS	Intermediate Symptomatic
λ (Lambda)	Wavelength
LC	Liquid Chromatography
LDA	Linear Discriminant Analysis

LDC	Linear Discriminant Classifier
MCT	Mercury Cadmium Telluride
MIR	Mid Infrared
MS	Mass Spectrometry
ν (Nu)	Frequency
ROS	Reactive Oxygen Species
RS	Ripening Stage
PC	Principal Component
PCA	Principal Component Analysis
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PP	Pest and Pathogens
PS	Pre-Symptomatic
SAR	Systemic Acquired Resistance
SERS	Surface Enhanced Raman Spectroscopy
SNR	Signal-to-Noise Ratio
SRS	Stimulated Raman Scattering
SVM	Support Vector Machine
TF	Transcription Factor
UAVs	Unmanned Aerial Vehicles
UF	Upper Fingerprint
VOC	Volatile Organic Compound

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Chapter 1: General Introduction

1.1 Introduction

Plants are essential for sustaining human life. As primary producers, plants harness the sun's energy, for subsequent distribution of chemical energy into the base of the food web (Pimm et al. 1991). Animal evolution is fundamentally linked to plant life and ensured that our relationship with plants would remain intimate and indispensable (Jordano et al. 2003). Successful cultivation of plants and the invention of agriculture was one of the most important milestones facilitating the development of complex civilizations; while the ability to produce and store excess food led us into a modern world away from the previous hunter-gatherer lifestyle of our ancestors (Diamond 2005). This transition and the accompanying manipulation of the natural environment, to meet growing horticultural demands over time, became increasingly challenging in the 20th century. The world population has been increasing more rapidly since the beginning of the 20th century (Figure 1.1). Presently the global population of approximately 7.5 billion people is expected to increase and exceed between 9.6 and 12.3 billion by the year 2100 (Gerland et al. 2014). Meeting the food demands of this fast population rise increases the pressure to produce more food, in a safe and sustainable manner to ensure food security and prevent destruction of the natural environment.

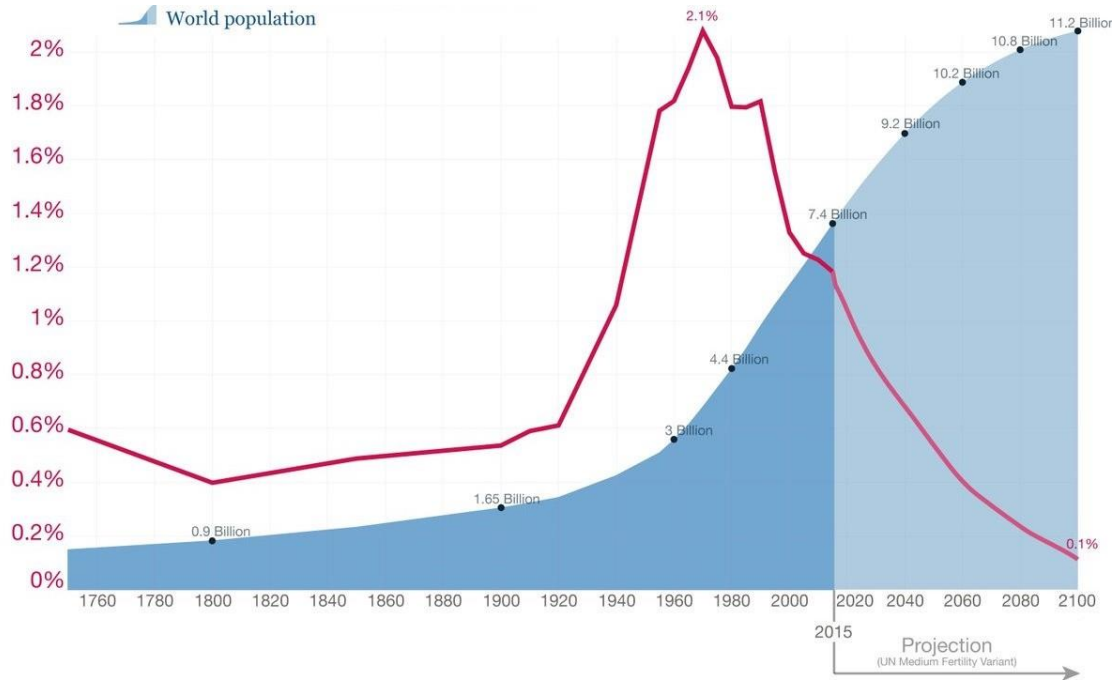

Figure 1.1 Global human population growth curve and rate of population increase from 1760-2015 including estimated future projections (from Roser and Ortiz-Ospina 2017).

As part of ensuring food security in line with the expected human population growth, new farming practices, to supersede more traditional ones, were developed in the 1950s as part of the 'green revolution'. The green revolution can be regarded as a set of practices aimed at increasing worldwide agricultural productivity through knowledge-exchange from fundamental plant research to applied horticulture (Evenson and Gollin 2003). Developments in the field of plant science have contributed to improved crop production through methods that increase both crop yield and quality (Premanandh 2011). These methods are frequently combined to attain satisfactory yield and quality of crops. Plant breeding, including trait selection (phenotypic and genotypic), is amongst the oldest and most exploited methods used in plant research (Roberts 1929). Identifying and breeding naturally resilient crops is considered favorable but is time intense and expensive (Mahlein 2016). To accelerate the natural selection process for favorable traits, a related strategy may be used. Genetic modification (GM) of crops has been used to generate crops that are resistant to environmental stresses, in addition to manipulating other traits that can potentially improve, for example, crop shelf-life, color and flavor (Qaim and Kouser 2013). Significant advancements have been made with GM crops, many of which have been developed for increased resistance to disease, pests, and pathogens (Birch and Wheatley 2005). While GM crops offer an alternative to increasing crop production and to lengthy selective breeding programs, public opinion, and thus marketability of GM crops can be a challenge. Through the relatively recent discovery of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR-Cas9), GM crops may be substantially improved, making available virtually any genetic target within the plant genome with increasing speed (Bortesi and Fisher 2015). This can be combined with other approaches including the precision control of the growth environments, where hydroponic systems and artificial growth mediums, irrigation strategies, nutrient supply and optimized lighting, allow crop production on both large and small scales. Despite these large technological strides, producing enough food to feed all of humanity remains to be achieved (Ray et al. 2013). Today's farming approaches combined with our population boom has led to patchy food security around the globe and predictions are suggesting that it will become even more challenging to meet future food demands (Ray et al. 2013). Food production and supply will therefore have to improve, due to the tremendous pressure to generate more food with less resources, less environmental impact, and concurrently addressing the large fraction of the population that is malnourished (IFPIRI 2017).

The ability to cultivate crop plants is at the heart of all food production and this ability can be threatened. Abiotic and biotic stresses both greatly influence plant growth and development affecting yield potential and actual quality of crops (Atkinson and Urwin 2012; Lara 2014). Figure 1.2 shows the effects of individual and combined stress induced by abiotic (drought) and biotic (nematodes) factors. This example illustrates the unpredictable effects of combined stress, which may combine in various ways to have observable or more subtle consequences from individual types of stress.

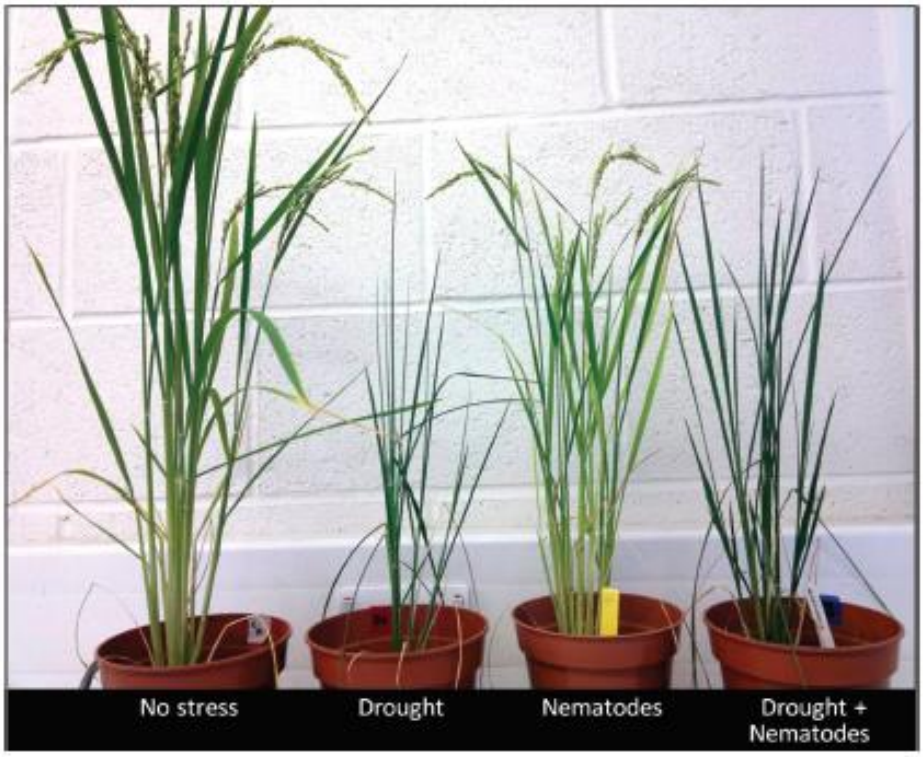

Figure 1.2 Effects of individual and combined stress induced by abiotic and biotic factors (from Atkinson and Urwin 2012).

While these are often regarded separately for plant and crop management purposes, plant response to abiotic and biotic factors overlap extensively (Kissoudis et al. 2014; Suzuki et al. 2014). Crop production from an ecosystem perspective thus considers all of the associated organisms (biotic) that live and interact with plants under a given set of environmental conditions (abiotic factors) (Suzuki et al. 2014). Harmful organisms that threaten crops are among the most important challenges to successful agriculture. Crop pests and pathogens (PP) are highly

diversified and include many classes of macroscopic and microscopic organisms. Unlike abiotic stress, PPs can adapt to changing conditions found during crop cultivation by evolving, re-distributing (migrating), and employing various attack strategies that can harm crops (Bebber 2015). Effects of PPs are therefore diverse ranging from mild losses to the destruction of virtually entire crops (Oerke 2006) (see also, Section 1.5). This diversity in PP strategy and resulting effects on crops, shows the importance of improving our understanding of PP-host interactions in the context of global food security. From a preventative standpoint it is equally important to be able to detect early signs of the presence of PP, ideally before plants are significantly compromised. Through human manipulation of the food web and increased homogenization of the biosphere, PPs have thrived in many instances, leading to the wide use of pesticides as countermeasures (Popp et al. 2012). However, due to the negative associations with pesticides, including public pressure, modern agriculture is moving towards a more precision approach aimed at utilizing technology for reducing pesticide application and integrating traditional and new solutions for crop protection (Barzman et al. 2015).

Crop protection refers to strategies for preventing the adverse effects caused by biotic and abiotic factors to ensure healthy crops (ECPA 2019). Modern crop protection is changing due to the evolving demands of agriculture worldwide; as such, the intricacy of adapting crop protection strategies to a changing world is shown by the involvement of science, enterprise, and the government (Popp 2011). Traditionally, crop protection has been associated with the use of pesticides; a broad category of chemicals aimed at treating various forms of PPs that cause plant disease (Popp et al. 2012). Pesticide use has become the focus of growing concern however due to the potential adverse effects of pesticides on the environment and human health when used in excess (Lechenet et al. 2014). It has become evident that pesticides are often used at levels much higher than needed, if needed at all, to attain specific yield thresholds (Lechenet et al. 2014; Vasileiadis 2017). Modern practices encompassed by precision crop protection, are thus favoring alternatives to pesticides, where efforts are being made to optimize pesticide productivity and minimize overuse.

Integrated pest management (IPM) is referred to as a holistic approach to crop protection that is sustainable and focuses on the prevention and treatment of plant diseases by combining methods that are cost effective and environmentally responsible (ECPA 2019). IPM is an evolving concept referring to a collection of measures intended to prevent significant negative effects on

crops by biological organisms through the sustainable use of best available plant protection products (Barzman et al. 2015). It entails methods developed as part of the green revolution, including considerations about crop rotation and field ecology, as well as more advanced state-of-the-art monitoring and information systems upon which decisions are made (Barzman et al. 2015) (see also, Sections 1.3 and 1.4.2). While rapid sensors monitoring environmental parameters are already routinely used on farming machinery (Pérez-Ruiz et al. 2015), the routine monitoring and detection of PPs is not currently part of the agricultural framework. However, sensor types are highly diverse, ranging from those that work at great distances (distal sensors) including satellites, aircrafts, and unmanned aerial vehicles (UAVs), to ground based sensors attached to tractors, handheld units, as well as sensors working at the cellular or molecular level (Mahlein 2016). Sensors capable of PP detection in practice, although scarce to date, are currently being developed (Mahlein 2016; Martinelli et al. 2014; Sankaran et al. 2010). Part of the reason for the lack of sensors for PP detection lies in the biological complexity and variability of plant-PP interactions. The early detection of plant diseases, caused by small or microscopic pests, is of paramount importance as these are among the most difficult to control (Williamson et al. 2007). Pre-symptomatic detection, whether direct detection of PPs, or indirect detection of the plant's responses, remains a key aspect of crop protection (Sankaran et al. 2010). Plant disease from initial exposure to PP, through disease progression, leading to reduced crop yields, are therefore research areas that require increasing attention (Mahlein 2016; Skolik et al. 2018b). Consequently, moving towards a more sustainable and precision crop production framework, requires technological solutions to better crop production.

Crop protection by way of IPM depends, at least in part, on effective sensors which provide the information necessary to facilitate pest detection. Sensors that do not damage plants during analysis are clearly favorable as they reduce sample preparation, facilitating intact analysis, thereby increasing measurement speed, and subsequent interpretation of data. Most of these sensors rely on interaction between plants with various ranges of electromagnetic radiation (Mahlein 2016).

Within the class of non-destructive sensors are spectroscopy-based methods relying on information gained by measuring the interaction between light and matter. Among these are vibrational spectroscopy methods including mid-infrared (MIR) and Raman spectroscopy (Baker et al. 2014; Butler et al. 2016). Applications of vibrational spectroscopy in plant research are still

limited, especially applications where the non-destructive nature of these techniques is conserved. Even so, the rapid development of the vibrational spectroscopy field into the plant and crop sciences is underway; emphasis is being placed on adapting these methods for application outside of the laboratory to more heterogenous systems found in commercial growing environments (Skolik et al. 2018b). Based on current investigations and with further research and development, vibrational spectroscopy will contribute to crop protection and thus aid in the improvement of food security worldwide by becoming established as a prominent sensor technology for plant science and horticultural applications in particular (Egging et al. 2018; Farber and Kurouski 2018; Skolik et al. 2018a; Yeturu et al. 2016).

1.2 Food security

Global food security remains a major challenge throughout the world. Factors influencing food security are multi-faceted and complex. Food security has been defined in the 1996 world food summit stating that "all people, always, have physical and economic access to sufficient, safe, and nutritious food to meet their dietary needs" (FAO 2016; Porter et al. 2014). Continuously supplying safe and nutritious food, in other words providing food security for a global population, has been unsuccessful in that a large proportion of the world's population is malnourished (IFPIRI 2017). Not only is the current food security deficit of concern, deficits in the production of major staple crops suggests that food security will not improve by 2050 (Ray et al. 2013). Required yield increases each year for crops of maize, rice, wheat, and soybean need an increase of approximately 2.4% annually to meet the global food demands projected for 2050 (Ray et al. 2013). In reality, these rates are at 1.6%, 1.0%, 0.9%, and 1.3% per year for maize, rice, wheat, and soybean, respectively; leaving potentially drastic shortcomings of these crops in coming years (Ray et al. 2013).

Figure 1.3 provides an overview of malnutrition worldwide. Especially affected are underdeveloped countries where food security is already scarce, but where most people affected by food shortages reside (IFPIRI 2017). Central Africa is especially affected by food shortages, while most developed nations have low enough rates of malnutrition to not be directly included. The greatest need for improvement of food security is therefore necessary in Africa and Asia.

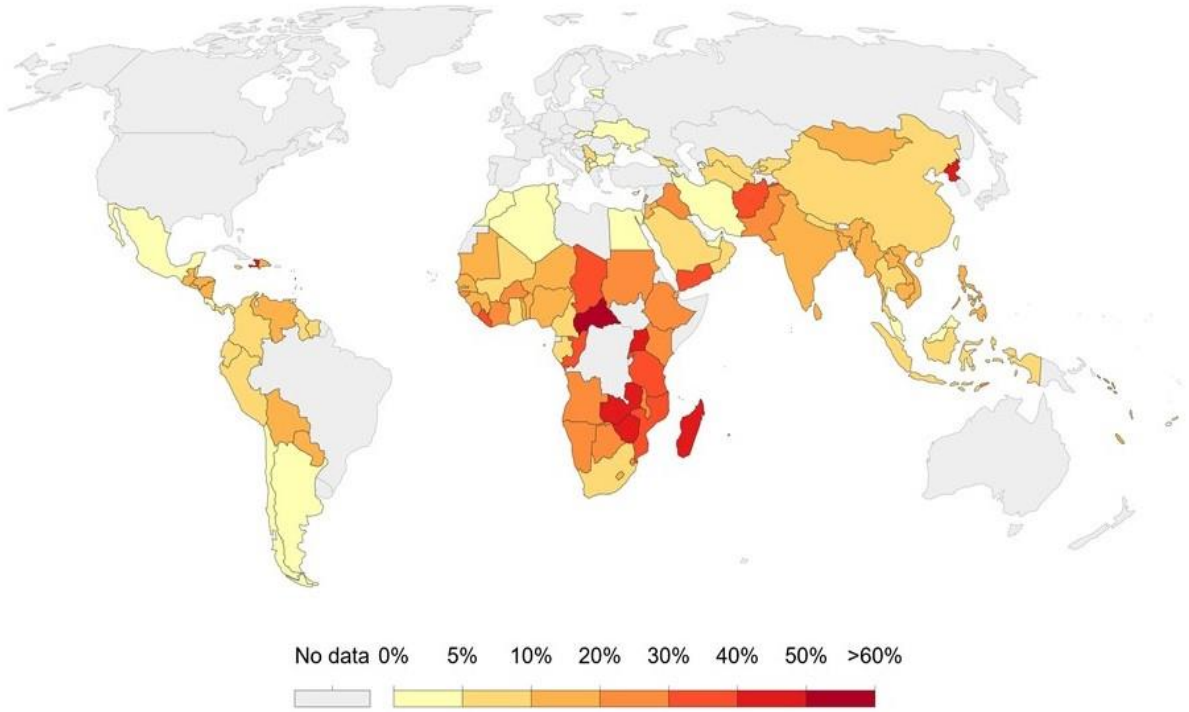

Figure 1.3 Malnutrition severity worldwide where data is available showing most malnourished people residing in developing African countries. (from Roser and Ritchie 2019; data from FAO 2018).

Whilst improved agricultural practices are likely to contribute to improved food security in developing countries the threat to food security in developed countries is largely due to post-harvest losses and consumer waste (Godfray et al. 2010). Developing countries have the potential to improve food security by adopting conventional solutions, which are commonplace in developed nations. Examples of these are potential for land clearing (crop production) and access to refrigerated transport (crop supply) (Godfray et al. 2010). Developed countries in contrast lose most food to waste, meaning a focus on losses at the post-harvest and consumer stages is needed (Godfray et al. 2010). Figure 1.4 gives an overview of food waste in developing and developed nations, showing the areas of food production/supply at which loss is incurred in the different countries, comparing the UK and US to developing nations.

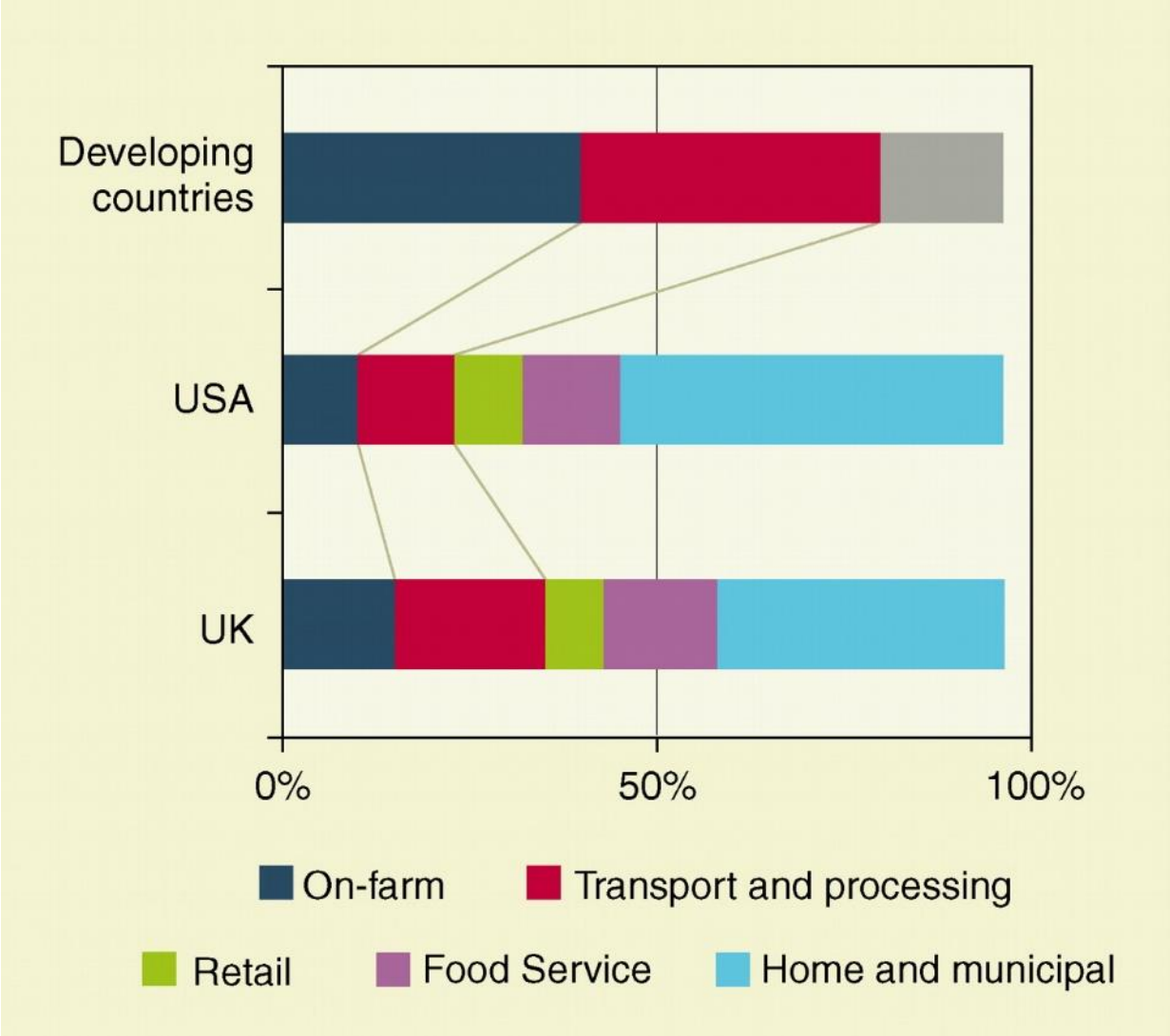

Figure 1.4 Food loss at different stages of food production/supply comparing differences between developing and developed nations (from Godfray et al. 2010).

The efficient and sustainable production of enough high-quality crops is critical to the achievement of unrestricted food security in both developing and developed nations. Solutions for improving food security in developed countries include high-tech concepts such as urban farms, rooftop gardens, vertical farming systems, and food sharing programs, aimed at optimizing crop production and reducing waste in areas not threatened substantially by food security (Despommier 2009; McClintock 2010). These high-tech concepts are, however, not available in developing nations, where on-farm loss and loss during transport are prominent (Figure 1.4). Recognition of the food security crisis has led to developments such as the increased distribution and repurposing

of nearly expired or otherwise unmarketable foodstuffs to prevent waste, which is becoming part of national policies in some countries such as France among others (Mourad 2015). Waste and loss reduction are integral parts of improving food security issues caused by growing pressures of population growth, land availability, and climate change. While current levels of food security are expected to deteriorate further, both developing and developed nations will be increasingly affected (Godfray et al. 2010; Ray et al. 2013).

PPs cause significant crop loss and thereby threaten food security (Oerke 2006). Apart from growing more crops, reducing losses to PPs is one of the most essential starting points for increasing agricultural productivity. This includes expanding our understanding of plant-environment interactions, while developing effective methods allowing precision scheduling of crop protection measures, including pesticide application where necessary. Plant interactions with PPs are therefore relevant for increasing crop production, firstly through the development of detection and monitoring systems, and secondly by expanding our knowledge of how PPs affect growth, development, yield, and quality of crops (Barzman et al. 2015; Lara et al. 2014).

1.3 Crop Protection

Crop protection is an essential component in the armory of countermeasures used against the effects of PPs in order to maximize crop productivity and hence food security. First and foremost, crop protection has been traditionally associated with the management of plant disease caused by PPs (Popp 2011; Popp et al. 2013). Modern crop protection is enveloped by the broader field of precision horticulture aimed at sustainably increasing food security (Gebbers and Adamchuk 2010). For centuries, farmers have relied heavily on techniques such as crop rotation, mixed crop planting, and the use of natural pest control such as predation, parasitism, and competition, for crop protection (Dayan et al. 2009). However, this changed along with the green revolution with the development of specialized agrochemicals including broad category of pesticides which have proved highly effective at increasing crop yields (Popp 2011). Modern farming has relied heavily on chemicals to prevent crop losses, despite the known harm caused by off-target effects, especially on human and environmental health (Bourguet and Guillemaud 2016). To reduce such effects and the overuse of crop protection products, while maintaining the crop yield advantages granted by such products, a more precision approach is frequently adopted. IPM is one such approach (Barzman et al. 2015) and precision crop protection through IPM can

therefore be adapted to essentially any environment and crop type(s) through the application of both conventional and specialized approaches. Development of autonomous farming equipment boasting innovative sensor systems, data processing centers, and actuation devices, can be highly beneficial as crop protection measures may be applied only when and where required, minimizing cost, environmental damage and exposure of farmers to dangerous chemical (Pérez-Ruiz et al. 2015). For this, crop protection is relying evermore on sensor data for real-time diagnostics.

1.4.1 Pesticide productivity

Crop yield models comparing theoretical crop yields to yields attained through the application of pesticides provide strong evidence of the importance of pesticides to the current level of food production (Popp 2011). Figure 1.5 shows the yield increases achievable through pesticide application. As farming has moved to more homogenous growing environments, crops have become more susceptible to PPs, resulting in the regular use of pesticides (Bourguet and Guillemaud 2016). Although the benefits of pesticides depend strongly on how they are used, evidence suggests that pesticide use is overall beneficial by contributing to crop protection and increasing crop yields (Popp 2011; Popp et al. 2012). However, overuse of pesticides is evidenced by often little reduction in yield despite no pesticide application at all and has resulted in a re-evaluation of pesticide productivity in recent years (Lechenet et al 2014; Singbo et al. 2015; Vasileiadis 2017). A major reason is that pesticide productivity is difficult to determine and can be overstated especially regarding ‘hidden’ or ‘shadow’ costs to ecosystems (Popp 2011; Singbo et al. 2015). Further, reducing pesticide use while maintaining the same levels of crop yield indeed increases pesticide productivity, which is why pesticide reduction is one of the high-priority targets in the quest for a sustainable agriculture (Vasileiadis 2017). Despite this it appears that pesticides will remain an essential part of crop production for the foreseeable future, especially if they significantly combat crop loss and help move towards the theoretical yield potential of crops (Popp 2011; Popp et al. 2012). Effects of microscopic pathogens (including viruses) are significantly reduced by crop protection, as seen by the crop yield gained (Figure 1.5). Nevertheless, these pests cannot be spotted without specialized detection methods and only become evident through symptoms, at which point plants may be irreversibly compromised. For timely use of crop protection measures, and to optimize pesticide use, safe and site-specific application of such is essential, irrespective of where and how they are employed.

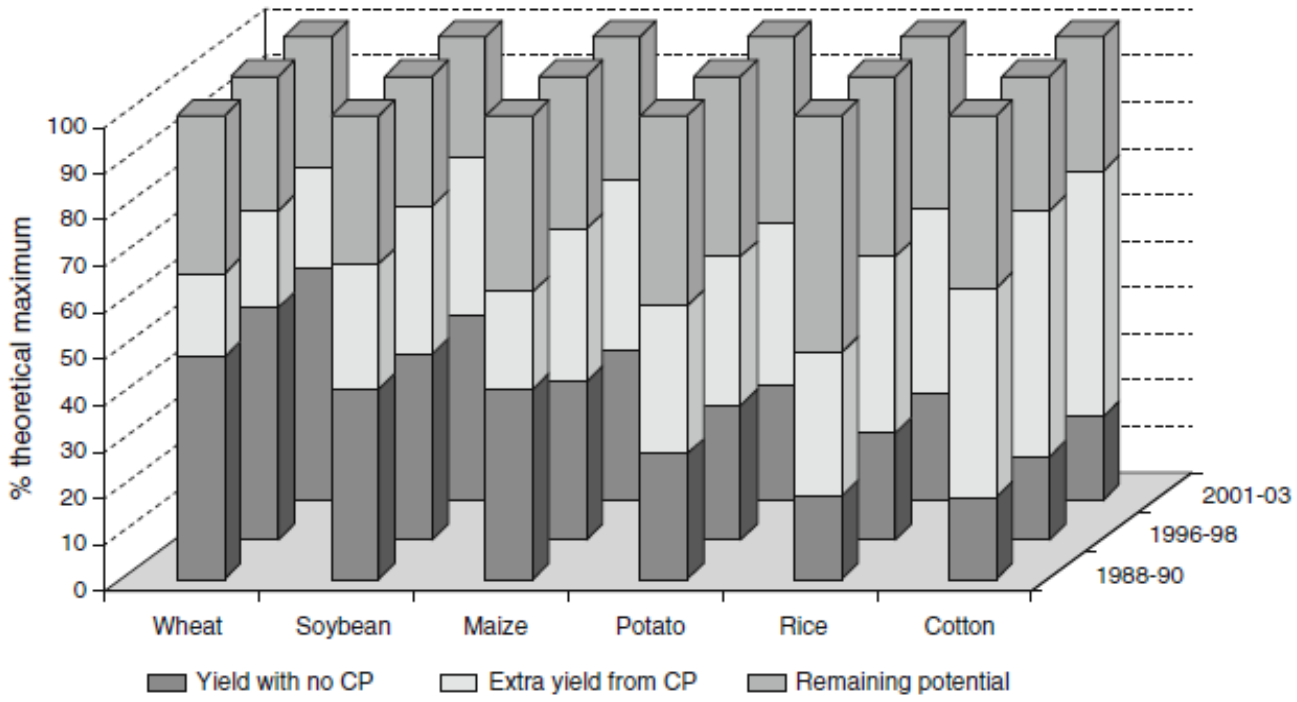

Figure 1.5 Pesticide productivity shown as crop yield benefits achieved by crop protection (from Popp 2011).

1.4.2 Integrated Pest Management

IPM is an important management tool for improving crop production. Conceptualized in the late 1950's by entomologists working with insecticides, IPM today encompasses virtually all aspects of plant protection (Barzman et al. 2015). This concept, while not new, may be appropriately tailored to facilitate the use of pesticides with other measures to maximize their effect and minimize human and environmental exposure. Several principles have been proposed to be integral to IPM (Barzman et al. 2015), divided into non-intervention and intervention related principles, including: prevention and suppression, monitoring, decision based on monitoring and thresholds, non-chemical methods, pesticide selection, reduced pesticide use, (pest) anti-resistance strategies, and evaluation (Figure 1.6). Table 1.1 provides a concise summary of each principle highlighted in Figure 1.6 showing the actions necessary for crop protection using the IPM approach.

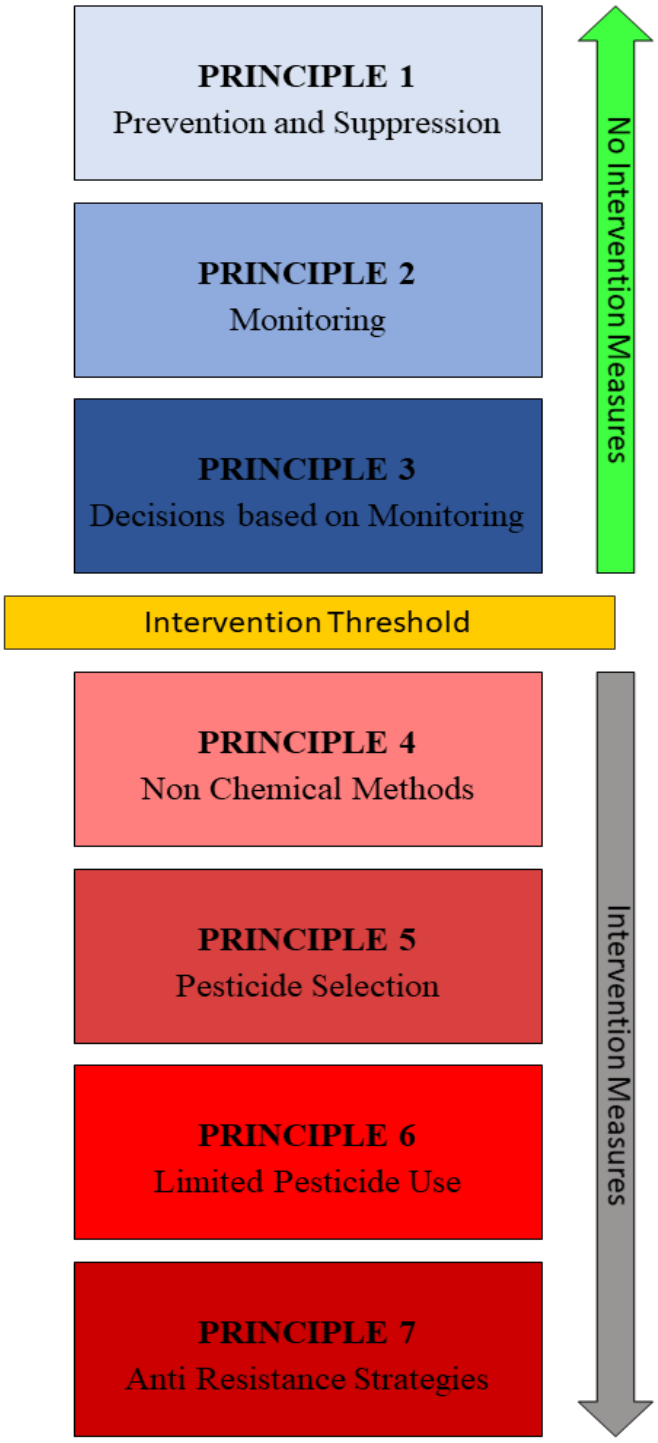

Figure 1.6 Principles of IPM showing the distinct principles relating to both non-intervention and intervention measures (from Barzman et al. 2015).

Table 1.1 Descriptive summary of IPM principles; see also Figure 1.6 (adapted from Barzman et al. 2015).

IPM Principle	Description	Action
Principle 1 Prevention and Suppression	The prevention and/or suppression of harmful organisms should be achieved or supported.	Crop rotation Use of adequate cultivation techniques (e.g. stale seedbed technique, sowing dates and densities, under sowing, conservation tillage, pruning and direct sowing). Use of resistant and or tolerant cultivars and standard or certified seed and planting material. Use of balanced fertilization, liming and irrigation and or drainage practices. Preventing the spreading of harmful organisms by hygiene measures (e.g. by regular cleansing of machinery and equipment). Protection and enhancement of important beneficial organisms (e.g. by adequate

		plant protection measures or the utilization of ecological infrastructures inside and outside production sites).
Principle 2 Monitoring	Harmful organisms must be monitored by adequate methods and tools.	Observations in the field as well as scientifically sound warning, forecasting and early diagnosis systems, where feasible, as well as the use of advice from professionally qualified advisors.
Principle 3 Decision-making	For harmful organisms, robust and scientifically threshold levels defined for the region, specific areas, crops and climatic conditions must be considered before treatments, where feasible.	Based on monitoring the professional user must decide whether and when to apply plant protection measures.
Principle 4 Non-chemical methods	Sustainable biological, physical and other non-chemical methods must be preferred to chemical methods if they provide satisfactory pest control.	Application of sustainable biological, physical and other non-chemical methods.

Principle 5 Pesticide selection	The pesticides applied shall be as specific as possible for the target and shall have the least side effects on human health, non-target organisms and the environment.	Application of a specifically and carefully selected pesticide versus generic pest control.
Principle 6 Reduced pesticide use	The professional user should keep the use of pesticides and other forms of intervention to levels that are necessary considering that the level of risk is acceptable, and they do not increase the risk for development of resistance in populations of harmful organisms.	Pesticide application of appropriately selected pesticide at minimal levels required to prevent crop loss (e.g. by reduced doses, reduced application frequency or partial applications).
Principle 7 Anti-resistance strategies	Where the risk of resistance against a plant protection measure is known and where the level of harmful organisms requires repeated application of pesticides to the crops, pesticide resistance needs to be considered.	Anti-resistance strategies should be applied to maintain the effectiveness of the products. This may include the use of multiple pesticides with different modes of action.
Principle 8 Evaluation	Crop protection measure evaluation, based on pesticide use, records, and on the monitoring of harmful organisms.	Check the success of the applied crop protection measures.

Globally, the implementation of IPM is limited by lack of experts and technical training, issues pertaining to research and outreach, and weak adoption incentives that have limited IPM implementation (Parsa et al. 2014). Consequently, compiling strategies with simple components (procedures, protocols, technical components, etc.) may increase the adoption of IPM. Principles 2 and 3 (monitoring and decision based on monitoring/thresholds) rely on accurate information to make decisions whether to intervene (Barzman et al. 2015). It is therefore highly likely that if effective sensors are developed, which are easy to use and implement in IPM strategies, crop protection will become more robust. The effective detection, identification, and quantification of PPs in the plant (population) to enable appropriate and precise crop protection remediation measures to be implemented thus remains a key area for improvement (Barzman et al. 2015; Mahlein 2016; Skolik et al. 2018b).

1.5 Pests and Pathogens of Crops

Since the beginning of agriculture, the yield potential of crops cultivated by humans has been affected by natural but harmful organisms including plants, animals, and PPs. Persistent PPs are regarded as one of the most significant biological influences affecting crop production (Oerke 2006; Bebber et al. 2014). Crop loss to PPs ranging from viruses to large animals and competitive plants is considerable at around 35%, although this is highly variable depending on the crop and PP (Oerke 2006; Oerke and Dehne 2004). It does nonetheless illustrate the gravity of crop loss to biological threats on a large scale. Macroscopic PPs, include large animal herbivores, insects and plants (weeds), whilst microscopic PPs include viruses, bacteria, fungi and microscopic animals (mites, nematodes, etc.). Whilst macroscopic PPs can be detected by eye and are thus easier to manage without the use of harmful agrochemicals (Oerke 2006), reducing the impact of microscopic PP is more challenging as they are more difficult to detect prior to incurring crop loss, and thus contribute to a minimum loss of 10% globally (Strange and Scott 2005). This is further compounded by the reduced efficacy of crop protection against microscopic compared to macroscopic PPs (Oerke 2006). Confounding this issue is the rapid expansion and adaptation of pathogens to new territories due to climate change and it is expected that the homogeneous distribution of pathogens across all crop areas will occur in the coming decades (Bebber 2015; Bebber et al. 2013; Juroszek and Tiedemann 2013). As a result, plant-PP interactions are of high

importance to plant pathologists and growers, because they involve complex biological interactions that occur at the molecular level (Lucas 2011). Respectively, the threat to crops posed by PPs is increasing and to counteract this, adaptable methods for pest detection and control, as part of IPM will be imperative.

1.5.1 Evidence of Crop Loss to Pests and Pathogens

Depending on the crop, annual losses can reach as high as 50%-80% for certain crops such as cotton and sugar beet (Chakraborty et al. 2011). Overall, loss potential is highest for weeds (34%) and lowest for viruses (5%), with animal pests (18%) accounting for just slightly more than pathogens (16%) (Oerke 2006). However, in the context of pesticide efficacy, crop loss due to weeds, animal pests, and pathogens (bacteria, fungi, viruses, and other microbes), are reduced 74, 39, and 37% respectively (Oerke 2006). Nevertheless, it is clear that pathogens present a unique challenge to crop protection, which is likely due to their microscopic nature. Therefore, reducing crop losses due to plant interactions with microscopic pathogens, bacteria, fungi, and viruses specifically, appear to offer the largest room for improvement. Consequently, increasing our understanding of plant-PP interactions, especially interactions with difficult to detect microscopic pathogens is fundamental to more precise crop production.

1.5.2 Plant-Pest and Pathogen Interactions

Understanding the molecular details of plant interactions with PPs will improve crop protection. PP have developed a broad suite of strategies for interacting with plants which are beneficial to the PP but potentially detrimental to plants (Vleeshouwers and Oliver 2014). Whether as food, habitat, or both, PPs interact with their plants in diverse ways to exploit weaknesses in the plant's biology (Atkinson and Urwin 2012; Boller and He 2009). In response, plants have adapted complex counter strategies as defense against PPs (Fujita et al. 2006). Plant-PP interactions are part of an evolutionary arms-race, where both the plant and invader struggle for survival (Boller and He 2009). Influences of PPs on plants may result in reduced fitness leading to lower crop yield or lead to massive crop loss through the manifestation of plant diseases. This is apparent in the context of variable crop loss depending on pest type, host, and environmental conditions (Oerke 2006). Crop production may therefore be affected directly, for example, through predation or plant disease, or indirectly for instance via habitat and resource competition (Miller 1994). Frequently,

plants are compromised through interactions with PPs, leading to reduced growth and development due to partitioning resources towards defense strategies.

Exposure to biotic and or abiotic stress triggers changes at the molecular, cellular, and physiological levels that overlap in complex ways to prevent harmful effects of PPs. Whether in combination of as individual stress types, both biotic and abiotic stress causes massive changes that begin through the induction of general stress responses shared between biotic and abiotic responses (Fujita et al. 2006; Kissoudis et al. 2014). Initially, stress is perceived by receptors specialized for certain types of stress (e.g. osmotic, pathogen) inducing signal transduction pathways that include hormone signaling, MAP kinase cascades, and the ROS network (Atkinson and Urwin 2012). Signal transduction leads to the altered activity of transcription factors (TF), which may be controlled further through post-translational modifications. Altered expression of stress linked TF (e.g. HSF, MYB, zinc-finger type TFs) leads to altered gene expression of defense response genes that include metabolite production, cell wall fortification, altered ROS distribution, and cell death (Atkinson and Urwin 2012) (Figure 1.7). Select defense responses in plants may become valuable targets for the detection of PPs in crop systems. Specifically, ROS and abscisic acid (ABA) have been shown to influence structures initially confronted with PPs, which include the cuticle, cell wall, as well as underlying epidermis (Atkinson and Urwin 2012; Fujita et al. 2006; Kissoudis et al. 2014). Here both ROS and ABA have been associated with defense against pathogens specifically and the fortification of the epidermis through callose deposition (ABA driven) and lignin-crosslinking (ROS linked) (Kissoudis et al. 2014). As these networks influence resistance to pathogens, being able to detect these changes initially may present pre-symptomatic targets for early disease detection by some sensor technologies.

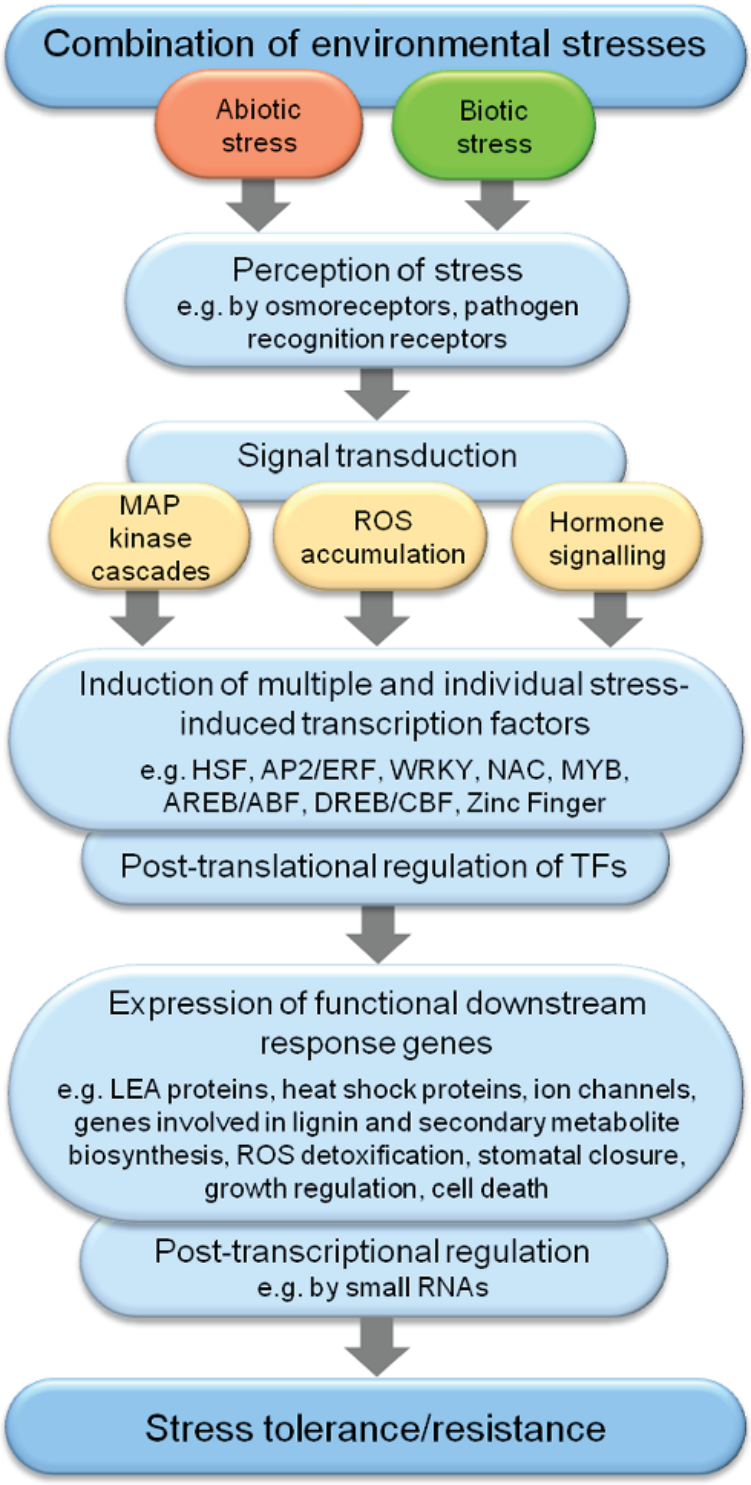

Figure 1.7 Overlap of plant response to abiotic and biotic stress at the cellular and molecular levels (from Atkinson and Urwin 2012).

1.6 Detection of Plant Pests and Pathogens

Precision agriculture relies heavily on detailed information obtained from sensors in the growing environment (Barzman et al. 2015). Early detection and identification of PPs is one of the first steps in negating the potentially catastrophic losses frequently associated with biotic stresses. There are many pest control measures available for macroscopic PPs. Although, there is no shortage of sensor types which may be developed for microscopic PP detection, identification, and or quantification (Mahlein 2016; Martinelli et al. 2014; Sankaran et al. 2010), microscopic pest detection remains technically challenging (Mahlein 2016). In particular, the variability of growing conditions means that sensors must be highly adaptable to changing environments. Due to the destructive nature of many existing approaches, the adoption of non-destructive sensors that do not rely on damaging plants/crops is pivotal. Additionally, sensor technologies must be compatible with farming equipment if these are to be used on a large scale (Pérez-Ruiz et al. 2015). Sensor development for the purposes of PP management therefore remains a challenging endeavor.

1.6.1 Sensor Types

A combination of sensor types is required to match the biological complexity of crop production. Sensors aimed at advancing crop protection and IPM fall into different categories depending on their features and identifying effective combinations will provide better safeguarding against PPs. Primary differences between sensors is sampling requirements, size, working distance, measurement range, spatial resolution, and acquisition time (Martinelli et al. 2014; Sankaran et al. 2010). Therefore, sensors must be appropriate for the level of biological organization under investigation, which range from the sub-cellular or cellular level to larger tissue sections such as leaves, all the way to the measurement of whole plots, fields, or even ecosystems (Mahlein 2016). Figure 1.8. shows the range of non-destructive sensor technologies available for crop protection and plant phenotyping. If a given sensor measures biological tissues, either at the whole plant, tissue, cellular, or sub-cellular levels, destructive sample preparation is often unavoidable. Aside from requiring skilled experts, methods requiring sample preparation are invasive, requiring sample manipulation, specialized workspaces, considerable preparation time, while providing limited amount of information regarding whole plant-environment interactions (Skolik et al. 2018b). This renders many approaches ineffective, specifically for *in vivo* analysis of intact crops in the field, or for use by growers. Correspondingly, techniques capable of rapidly

generating tissue, cellular, and or biochemically specific profiles of plants non-destructively would be immensely valuable to farmers, as well as plant and crop scientists. Effective sensors will then provide discriminatory information for detecting either subtle effects, such as ones on the cellular level including plant defense biochemistry, or larger scale changes, such as identifying diseased areas at the whole plant, field, or ecosystem scale. Various sensor types aimed at disease detection or phenotyping may be suitable for both purposes. Combining sensor types is also common, especially for optical sensors that operate on similar principles.

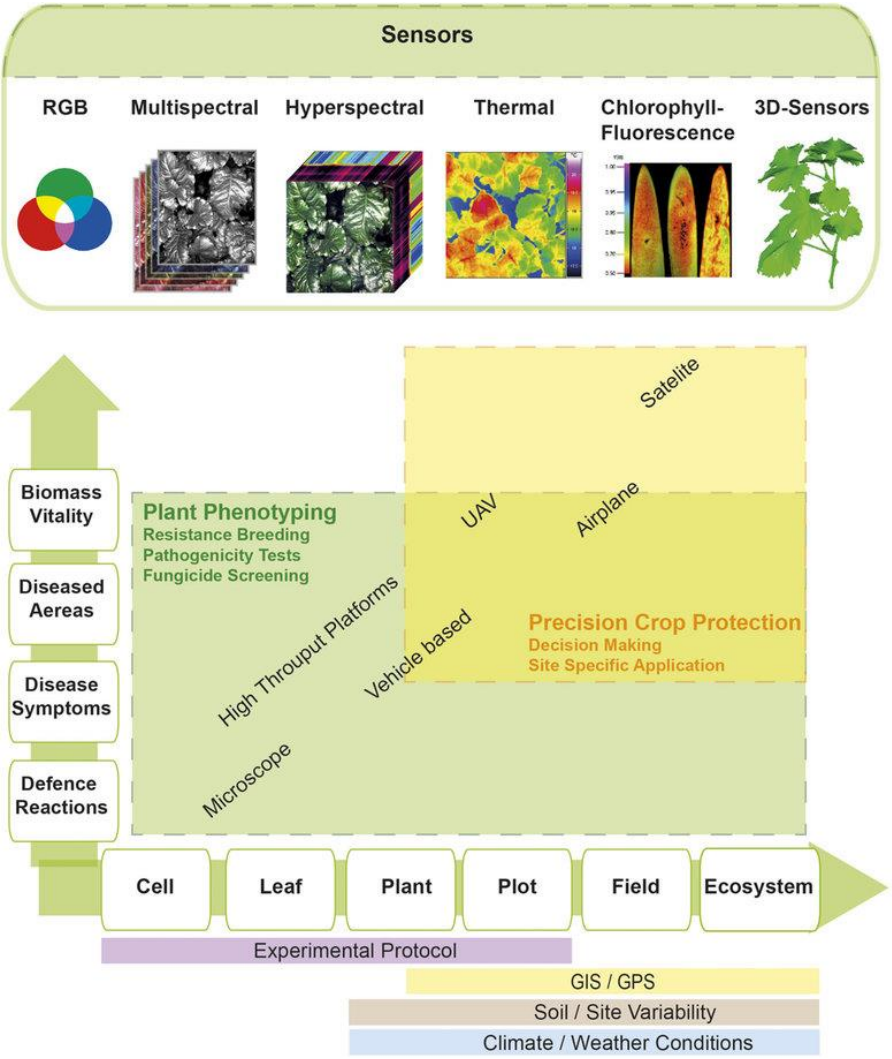

Figure 1.8 Overview of non-destructive sensor technologies for crop protection (from Mahlein 2016).

1.6.2 Non-Destructive Sensors

Sensors that do not damage plants and plant products are essential to avoiding crop loss. Non-destructive sensors work on the principle of chemical, mechanical, and or structural changes (Hahn 2009). These sensor types can be used for disease detection and identification/quantification of the associated PPs, as well as for other crop protection strategies such as phenotyping (Mahlein 2016). Many non-destructive sensors specifically for disease detection depend on measuring the interaction between electromagnetic radiation and the sample, with an emphasis on remote sensing and autonomous evaluation of these technologies for warning and monitoring schemes in plants and crops (Martinelli et al. 2014).

1.6.3 Spectroscopy Based Sensors

Spectroscopy-based sensors are among commonly used sensors for disease detection (Martinelli et al. 2014; Sankaran et al. 2010). Imaging and non-imaging spectroscopic techniques using visible, infrared, fluorescence, multiband, and ultraviolet ranges of electromagnetic radiation are particularly promising tools because they are adaptable, efficient, and cost effective (Martinelli et al. 2014; Sankaran et al. 2010; Hahn 2009). With a high demand for non-destructive methods, the rapid and disease-specific information gained from these sensors has been used for disease detection particularly in the pre-symptomatic stages both pre and post-harvest (Martinelli et al. 2014). These sensors rely on changes in plant tissues that alter their optical properties due to modifications of chemical components and structural characteristics (Mahlein 2016). Effects of crop PPs generally alter the optical properties of plant tissues often in the pre-symptomatic stage of plant disease (Mahlein 2016). A major advantage is that these sensors can be easily made operational with minimal retooling and can be incorporated into farming machinery for autonomous disease detection and monitoring (Pérez-Ruiz et al. 2015). In recent years infrared spectroscopy, specifically in the MIR region, have evolved to become strong contenders for commercial applications (Butler et al. 2015; Ord et al. 2016; Yeturu et al. 2016). Their prominence in this field in conjunction with further development will help usher in a new wave of sustainable science aimed at precision crop protection leading to decreased losses and better crop production.

1.7 Vibrational Spectroscopy

Spectroscopy is based on measuring interaction phenomena between electromagnetic radiation and matter. Vibrational spectroscopy relies on measurements of the interaction between IR radiation and matter (Figure 1.9).

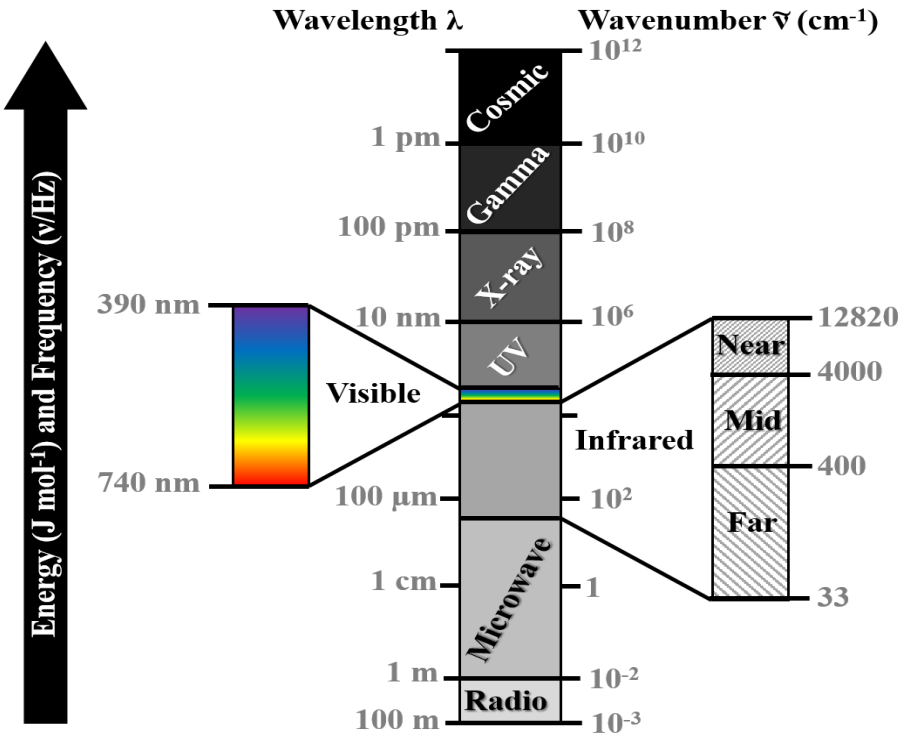

Figure 1.9 The electromagnetic spectrum showing the infrared wavelengths used for vibrational spectroscopy and adjacent visible range expanded for comparison (from Butler 2016).

Electromagnetic waves having wave-particle duality, contain electric and magnetic components and possess properties of discrete particles (photons) and waves (Andrews 2014; Stuart 2004). The physicist Max Planck initially described the relationship between photon energy I, wavelength (λ) and frequency (ν) of electromagnetic waves. Planck's constant h relates individual photon energy in the context of wave properties, where λ is inversely proportional to the frequency ν (i.e. 1/ λ) of the wave, leading to the simplified equation $E = h\nu$. MIR vibrational spectroscopy relies on the phenomenon of chemical bond vibration. Vibration in this case refers

to molecular energy levels higher than the ground-state (Andrews 2014; Stuart 2004). IR photons are absorbed by molecules if the photon energy exactly matches that needed to induce bond vibration, or the transition from ground to elevated electronic energy levels (Andrews 2014). Energy in the MIR matches vibrations of biochemical functional groups present in biological samples; energy transitions of vibrational spectroscopy are shown in Figure 1.10 (Baker et al. 2014). Shown transitions show that energy changes associated with MIR spectroscopy (red box) are lower than energy associated with Raman transitions (green box). The higher laser energy used by Raman instruments compared to IR spectrometers, leads to higher energy transitions that cannot be measured directly (virtual states) and are exceptionally rare (Butler et al. 2016).

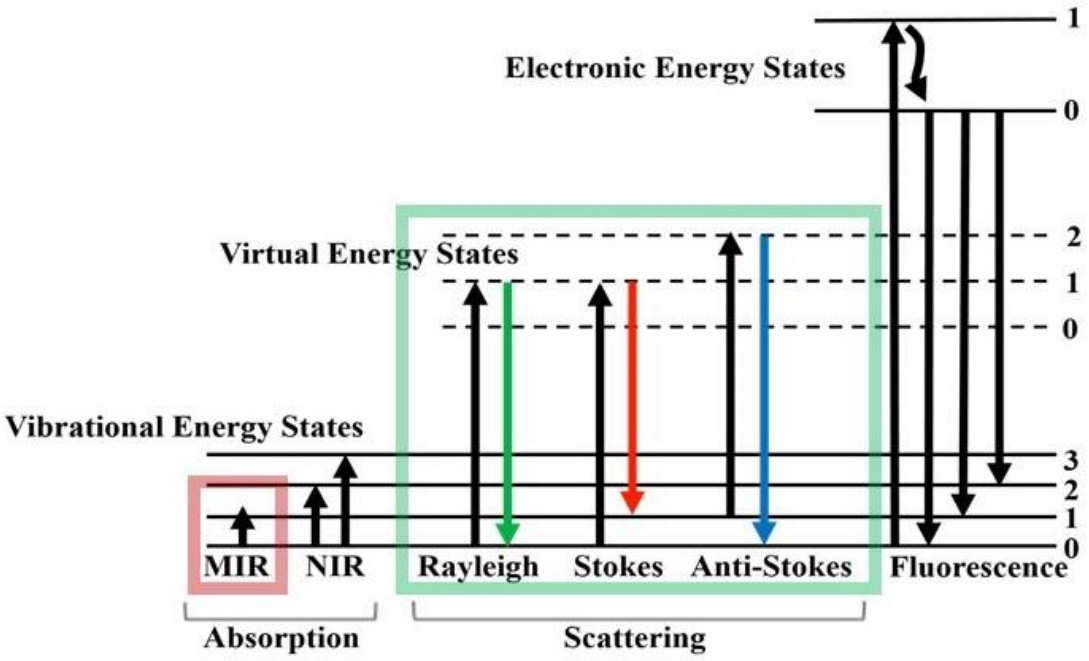

Figure 1.10 Electronic transitions associated with light absorption and scattering measured using MIR vibrational spectroscopy (red box) and Raman spectroscopy (green box), respectively (adapted from Baker et al. 2014).

Molecular bond vibrations can occur due to different light-matter interactions. These interactions include absorption, transmission, and reflectance, which are the fundamental interactions pertaining to IR spectroscopy (Andrews 2014). During the Raman phenomenon, light is scattered either elastically, or inelastically, where the IR radiation is unaltered and altered

respectively. As with all systems, energy conservation holds, meaning the energy of the IR source beam is accounted for by these interactions; all energy lost to absorption, transmission, reflectance, and scattering is equivalent to the source beam. These fundamental interactions rest on the principle that MIR electromagnetic radiation supplies the energy to induce electrons to migrate to energy levels higher than the zero-point energy (Baker et al. 2016; Sheppard 2006). Discrete energy levels imply that molecules have a discrete set of vibrational modes, depending on the bonding configurations present. In general, linear molecules will exhibit $3N - 5$, whereas for non-linear molecules $3N - 6$ normal vibrational modes occur; N is the number of atoms in the molecular arrangement (Andrews 2014). This discrepancy is because linear molecules have one less dimensional axis about which to rotate. One of the simplest examples is water (H_2O), which is a non-linear tri-atomic system leading to [3(3)-6 = 3] vibrational modes including symmetric and asymmetric O-H stretch, as well as the scissoring mode (Figure 1.11) (Andrews 2014; Du et al. 1993).

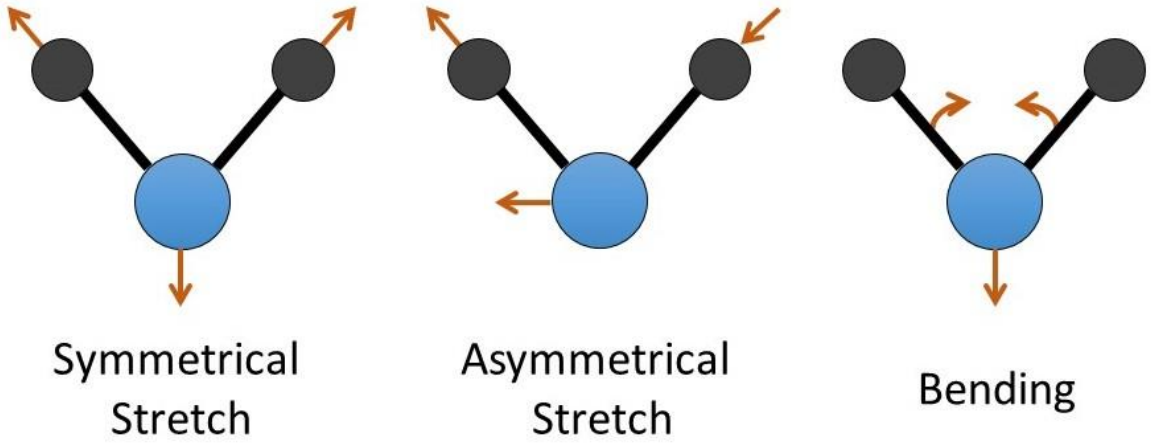

Figure 1.11 Three vibrational modes of water as determined by the formula 3N-6 for non-symmetrical molecules.

1.7.1 Infrared Spectroscopy

Modern IR spectroscopy became an analytical staple with the development of the Michelson Interferometer combined with Fourier transformation (FT). When measuring the absorption of light by a sample, the IR spectrum of a given material is unique. IR light absorption results in a change of the dipole moment of a molecule within the sample (Andrews 2014). As

seen in the vibrational modes of simple systems such as water, molecules that show strong symmetry or do not possess dipoles will not be easily interrogated using IR spectroscopy. However, the abundance of atoms and unique configurations present in even simple organic molecules, avoids this limitation (Andrews 2014). According to the Beer-Lambert Law, absorption is often linear over the defined measurement range making quantitative analysis possible, where absorption is proportionate to concentration (Kocsis et al. 2006; Swinehart 1962). The development of the Michelson interferometer and combining it with the FT made substantial improvements with regards to spectral acquisition speed and quality (Bracewell and Bracewell 1986). Vibrational spectroscopy, MIR spectroscopy and Raman spectroscopy, has been used increasingly used in the plant and crop sciences (Butler et al. 2015, 2017; Egging et al. 2018; Farber and Kurouski 2018; Skolik et al. 2018a; Yeturu et al. 2016).

1.7.1.1 Instrumentation

Common Fourier transformed IR (FTIR) spectrometers are composed of fundamental components including an IR source, interferometer, and detector. These components can be combined in various ways for specialized applications. The development of the FTIR spectroscopy permits the measurement of all IR wavelengths simultaneously and is a major milestone that has made FTIR such a widely used analytical method. The Michelson interferometer at the heart of the FTIR spectrometer (Figure 1.12) comprises two mirrors, one fixed and one mobile, as well as a beam splitter. IR light hitting the beam-splitter is focused upon both the fixed and mobile mirrors; when the two waves reflect towards the beam-splitter, they cancel each other out when at equivalent pathlengths. Moving the mirror, and thus changing the pathlength, produces waves of different phases resulting in an absorbance intensity as a function of distance and time (speed) of the mobile mirror; this is called an interferogram (Roddier and Roddier 1987). Applying the FT algorithm to the interferogram generates the wavenumber (frequency) absorbance (intensity) spectrum (Malacara and Servin 2016).

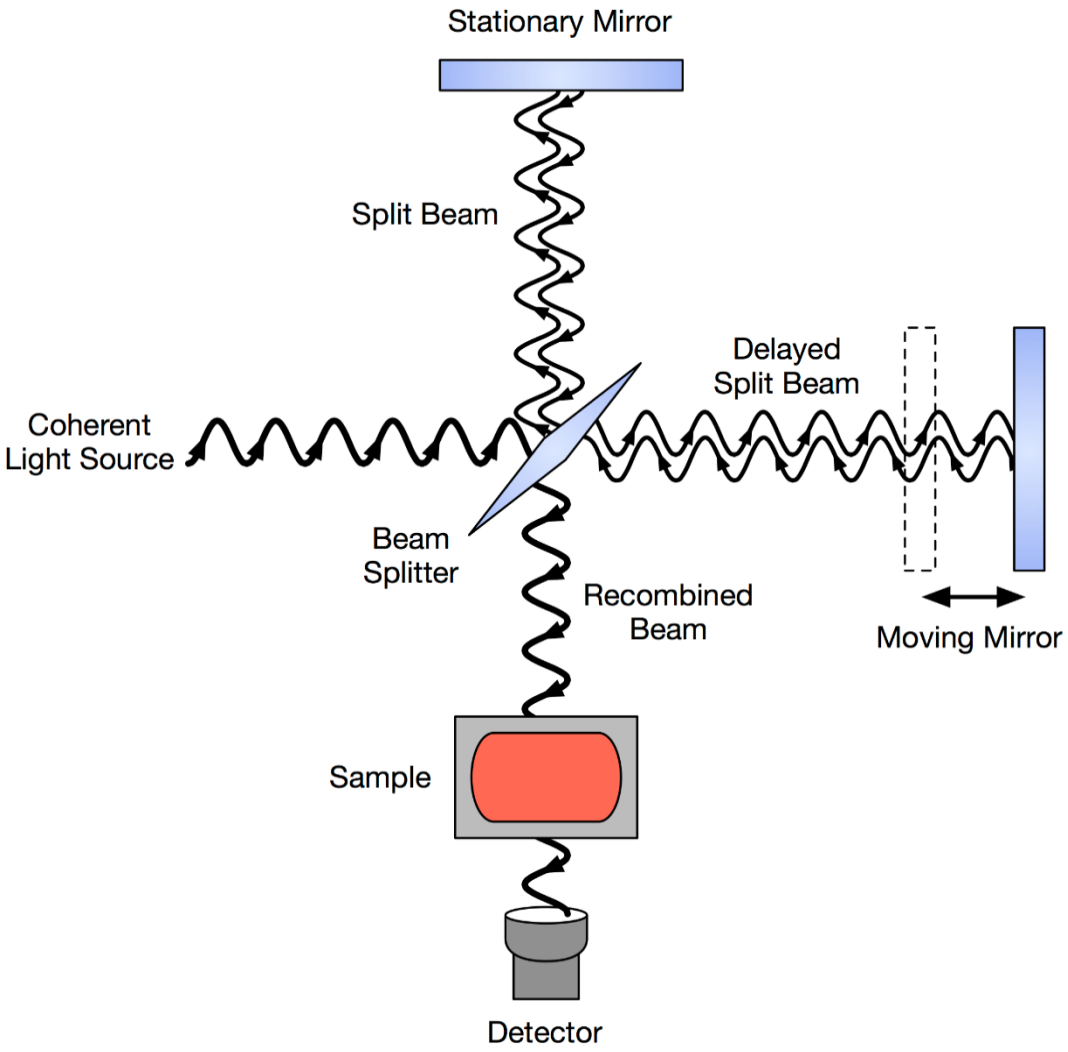

Figure 1.12 Schematic of a modern Michelson Interferometer used in modern MIR spectrometers.

Light sources for IR spectroscopy typically emit polychromatic light in the region 4000-400 cm^{-1}. This range of the IR spectrum corresponds to the fundamental vibrations of biological molecules within cells and tissues (Baker et al. 2014; Martin et al. 2010). Modern IR sources use silicon carbide rods that produce adequate light intensity for IR measurements. Also termed globar sources, these are the most commonly used for benchtop instruments. For light sources producing more intense IR radiation, quantum cascade lasers or synchrotron radiation are also available. Quantum cascade lasers can acquire spectra of superior quality over globar sources (Yeh et al. 2014). Synchrotron radiation, although more difficult to use in practice, can be several orders of magnitude brighter than conventional light sources (Miller and Smith 2005). Ultimately, the spatial resolution is constrained by the diffraction limit of the IR light. While high intensity lasers are

used to improve spectral quality and spatial resolution thereby providing more detailed molecular level information, their application is often reserved for specialized applications (Butler et al. 2017).

Detectors measure areas from the macro to the microscopic level, on a point-by-point basis or as an array. Macroscopic spectral acquisition can be viewed as one large pixel defined by the measurement area and falls into the category of point spectra (Chan and Kazarian 2016). Smaller microscopic point spectra can be obtained by using micro-spectroscopy, where spectra are acquired from microscopic regions as points, lines, or areas. Point spectra are detected by single element detectors. Many point spectra can be combined to form an image of a desired sample region, or a focal plane array (FPA) detector can be used to acquire IR signals from various points simultaneously (Chan and Kazarian 2016). FPA detectors can form diverse arrays containing hundreds of pixels with resolution down to the sub-micrometer range (Baker et al. 2014). Rapid acquisition using FPA detectors may in turn reduce the signal-to-noise (SNR) ratio (Baker et al. 2014). Detectors however place large time demands on high resolution line or area spectral acquisition for imaging or mapping applications. Commonly used detectors for macro or micro measurements include deuterated triglycine sulphate (DTGS) and mercury cadmium telluride (MCT) detectors. For most applications these offer adequate sensitivity, where MCT detectors are more sensitive but also operate at low temperatures requiring liquid nitrogen, compared to DTGS detectors that work at ambient temperatures (Miller and Smith 2005; Stuart 2004).

1.7.1.2 Sampling Modes

Three main sampling modes predominate in IR spectroscopy: transmission, transflection, and reflectance (Figure 1.13). These sampling modes may be combined with microscopes such that a high spatial resolution can be achieved.

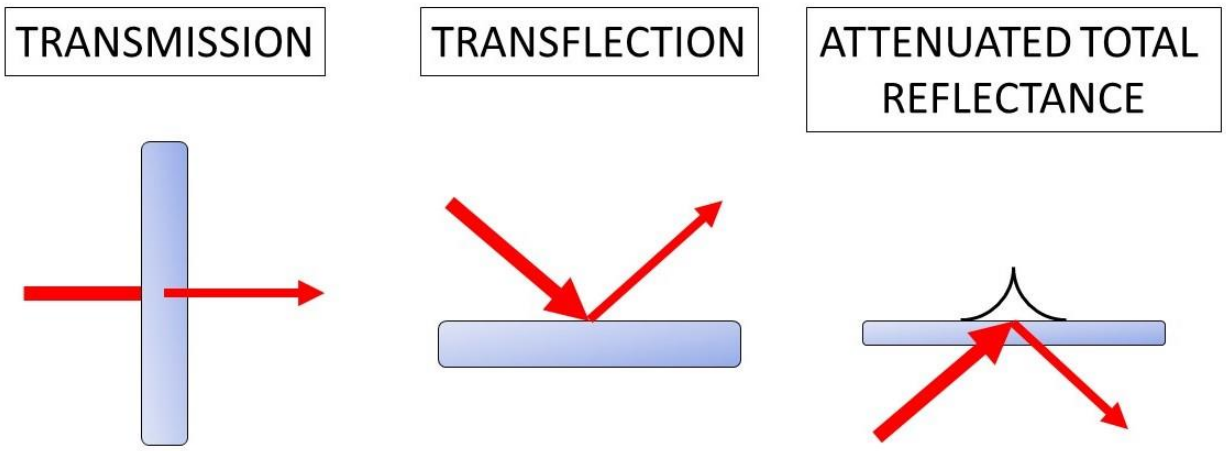

Figure 1.13 Three main sampling modes of IR spectroscopy; transmission, transflection, and ATR.

Transmission spectroscopy measures an IR beam that passes through the sample. Samples must therefore be contained on an IR transparent substrate. For this, slides made from barium fluoride (BaF_2) or calcium fluoride (CaF_2) are commonly used (Hahn 2014). In contrast to transmission measurements, transflection measures the IR beam after it has passed through the sample but is bounced back by an IR reflective material, rather than one which is transparent to IR radiation. The effect is that the IR beam essentially passes through the sample twice before returning to the detector. Transmission and transflection measurements however have drawbacks with regards to sample thickness and preparation. Both techniques are limited by the sample thickness for different reasons; transmission measurements are limited because the sample needs to be thin enough for the IR beam to pass through the sample. Transflection measurements are subject to the electric field standing wave effect, and therefore have a lower and upper thickness limit (Filik et al. 2012). Strong IR activity by water is another significant problem with IR measurements. Excess water can saturate the detector and make spectral acquisition difficult or impossible. As a result, samples must be dehydrated or fixed prior to spectroscopic analysis (Faolain et al. 2005). However, fixatives such as paraffin, formalin, or ethanol can significantly impact the IR signature, confounding interpretation of spectral data.

A further sampling mode better able to manage the effects of water and sample thickness is attenuated total reflectance (ATR) IR spectroscopy (Kazarian and Chan 2013). ATR-FTIR employs an internal reflection element (IRE) that makes direct contact with the sample and

precisely controls the measurement depth of the IR beam into the sample. This sampling mode drastically reduces the limitations of water content and sample thickness. Conventional ATR-FTIR is performed in macro mode without the use of microscopic optics. The IRE is a highly refractive material for which diamond, germanium, or zinc selenide are routinely used. These materials, along with the defined critical angle of the IR beam to the surface, ensure that the incident radiation is totally internally reflected (Kazarian and Chan 2013). Total internal reflectance produces an evanescent wave at the IRE-sample interface, where IR absorption by the sample attenuates the IR beam producing an absorbance spectrum. Because various materials are available for the IRE, depth of penetration of the beam into the sample may be a consideration if the sample is sufficiently thin, in which case an IR reflective substrate may be appropriate. The depth of penetration depends on the angle of incidence of the IR beam, as well as the refractive indices of sample and IRE respectively (Chan and Kazarian 2016). Despite the described differences, all sampling modes rely on chemical bond vibrations that alter the IR beam energy.

1.7.2 Raman Spectroscopy

Raman spectroscopy is a complementary method to IR spectroscopy sharing many principles. Named after C.V. Raman and K.S. Krishnan (1928), Raman spectroscopy utilizes a change in light energy after scattering referred to as inelastic light scattering. Although most light is elastically scattered, also termed Rayleigh scattering, Raman scattering in contrast occurs very minimally, where approximately 1 out of 10^8 photons are Raman scattered (Butler et al. 2016). These scattering phenomena are shown in Figure 1.14. Theoretically, the incident IR radiation can be excited (increase in energy), also known as Stokes scattering, or lose energy to the material leading to anti-Stokes scattering. Stokes-Raman is measured as the lower probability event of anti-Stokes scattering makes it difficult to acquire adequate signal strength, although there are ways of measuring anti-Stokes scattering using specialized approaches described elsewhere (Butler et al. 2016). Raman measures the phenomenon of molecular polarizability; this refers to the distortion of electron density around a molecule due to incoming energy (Heller et al. 1982). This change in the electron density distribution is considered a vibration.

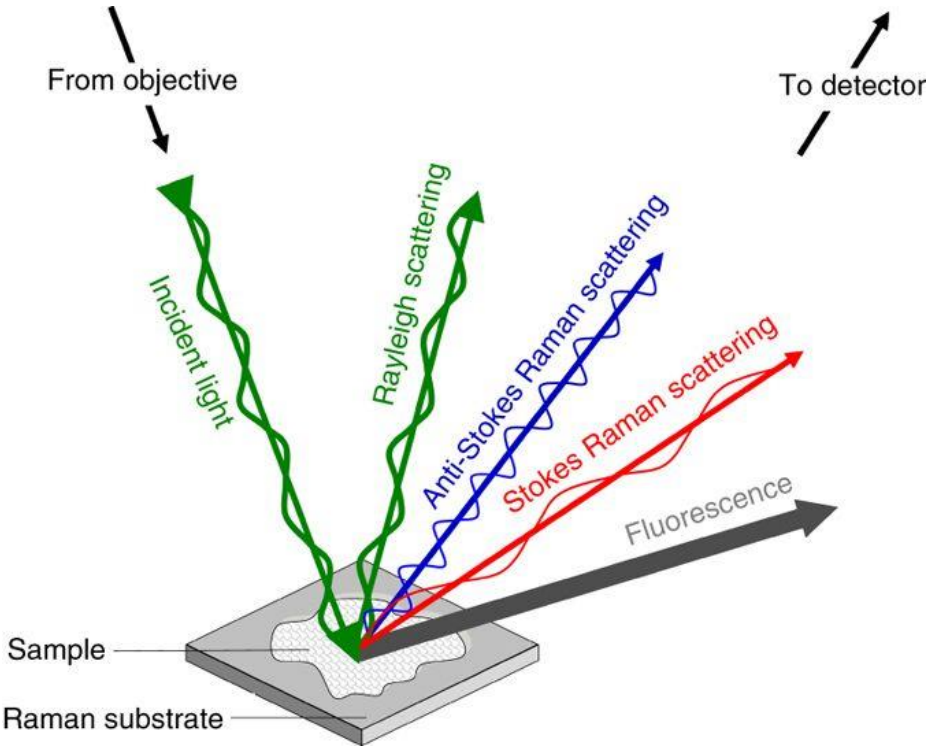

Figure 1.14 Scattering phenomena exploited by Raman spectroscopy showing Rayleigh, Anti-Stokes Raman, and Stokes Raman scattering, as well as the competing effect of fluorescence (from Butler et al. 2016).

In contrast to molecules having a dipole, polarizable molecules may or may not display changes in their dipole moment leading to different activities with regards to IR absorption and Raman scattering. As with IR spectroscopy, most of the instrumentation applications translate to Raman spectroscopy as well, with moderate differences in instrumentation (Butler et al. 2016; Baker et al. 2014; Martin et al. 2010).

1.7.3 Spectral Data Analysis

A spectrum contains an abundance of biochemical information by which to identify and discriminate biological samples. Spectral data is highly complex containing potentially hundreds of variables. High dimensionality of spectral datasets means that sophisticated analysis methods are often required to extract the information of interest. Chemometrics encompasses the data analysis methods used to handle IR spectral datasets (Morais et al. 2017; Trevisan et al. 2012). Many approaches for data handling and processing exist and depend on the experimental design;

nevertheless, several aspects are common to most approaches. Generally, raw spectra undergo baseline correction and normalization, together termed pre-processing account for confounding factors during spectral acquisition (Martin et al. 2010). Following pre-processing, spectra undergo either relatively simple analysis such as direct comparisons, univariate analysis, or characterization of spectral peaks. If samples are very similar or the natural sample heterogeneity is more pronounced than potentially subtle or masked differences that are class dependent, multivariate analysis may be employed. In the biological context, multivariate analysis is often necessary due to the biological complexity of the samples and the potentially underlying effects that require more advanced computational analysis.

1.7.3.1 Pre-processing

Spectral data pre-processing involves range selection, baseline correction, and normalization to account for confounding factors associated with biological materials. An IR spectrum contains the biological information of interest, which is influenced by differences in sample replicates, substrate, environmental interferences, including instrument and atmospheric differences (Morais et al. 2017; Trevisan et al. 2012). Such confounding factors must be accounted for before spectra can be compared to one another. Fundamental pre-processing steps including baseline correction and normalization approaches are introduced, but further reading is encouraged for a more detailed explanation.

Baseline correction is applied to account for spectral artefacts that need to be corrected for prior to subsequent analysis. Effects influencing spectral baselines for IR compared to Raman differ and generally require different baseline corrections respectively. IR spectra are affected mainly by the oscillating baseline that occurs due to light scattering (Bhargava et al. 1998). Differences in light scattering make it unreliable to compare IR spectra without baseline correction. In comparison, Raman spectra are affected by variable baselines caused by the underlying substrate and autofluorescence (Butler et al. 2015). For example, sloped Raman baselines are indicative of fluorescence present specifically in plant tissues (Butler et al. 2015, 2016). To correct for baseline aberrations, differentiation and rubber-band-like correction are almost exclusively used for IR spectra, while polynomial baseline correction is preferred for Raman spectra (Baker et al. 2014; Butler et al. 2016).

Polynomial baseline correction fits an n^{th}-order polynomial to the spectrum, which is

subtracted from the original Raman spectrum (Leger and Ryder 2006). Piecewise polynomial fitting has recently been proposed as an improvement for determining the polynomial degree needed for appropriate baseline correction (Hu et al. 2018). A calculus-based approach to spectral baseline correction is differentiation; this changes the fundamental shape of the original spectrum to either the first or second derivative spectra (Martin et al. 2010). First order differentiation results in spectral peaks to become zero and exaggerating more subtle spectral features often present in IR spectra (Baker et al. 2014). Second order differentiation relates to the curvature of the original spectral function but similarly to first-order differentiation accentuates spectral features potentially not evident in the original IR spectrum (Baker et al. 2014). Due to the introduction of peaks through differentiation, the SNR may also decrease, making noisy Raman spectra incompatible with this pre-processing step as the number of peaks resulting from differentiation can increase substantially (Martin et al. 2010). Rubber-band-like correction uses polygonal lines to find areas within the spectrum that are convex and to be subtracted from the baseline (Trevisan et al. 2012).

Following baseline correction, spectra are normalized to account for sample thickness and differences in light absorption and scattering. Inevitable differences in sample thickness or contact pressure in the case of ATR-FTIR, can potentially lead to slight differences in light absorption and scattering. Again, various normalization steps are available. Spectra may be normalized to a consistent spectral feature such as a consistent peak as demonstrated by using the amide I or amide II peak present in many biological materials (Baker et al. 2014; Martin et al. 2010). Common pre-processing steps for IR spectra include baseline correction and normalization (Figure 1.15). Rather than normalizing across the whole wavenumber range, peak normalization uses a single peak to achieve this (Martin et al. 2010). When it is unclear or when consistent peaks are unavailable, normalization across the entire wavenumber range used may be favorable. Here approaches including max-min and vector normalization are effective. Min-max normalization is simple, setting the minimum intensity of the spectrum, or specific region to zero and then scaling the maximum intensity to one (Lash 2012). Vector normalization works by finding the square root of the average spectral intensity squared, leading to spectra where the sum of squares of all intensities equals one (Lash 2012).

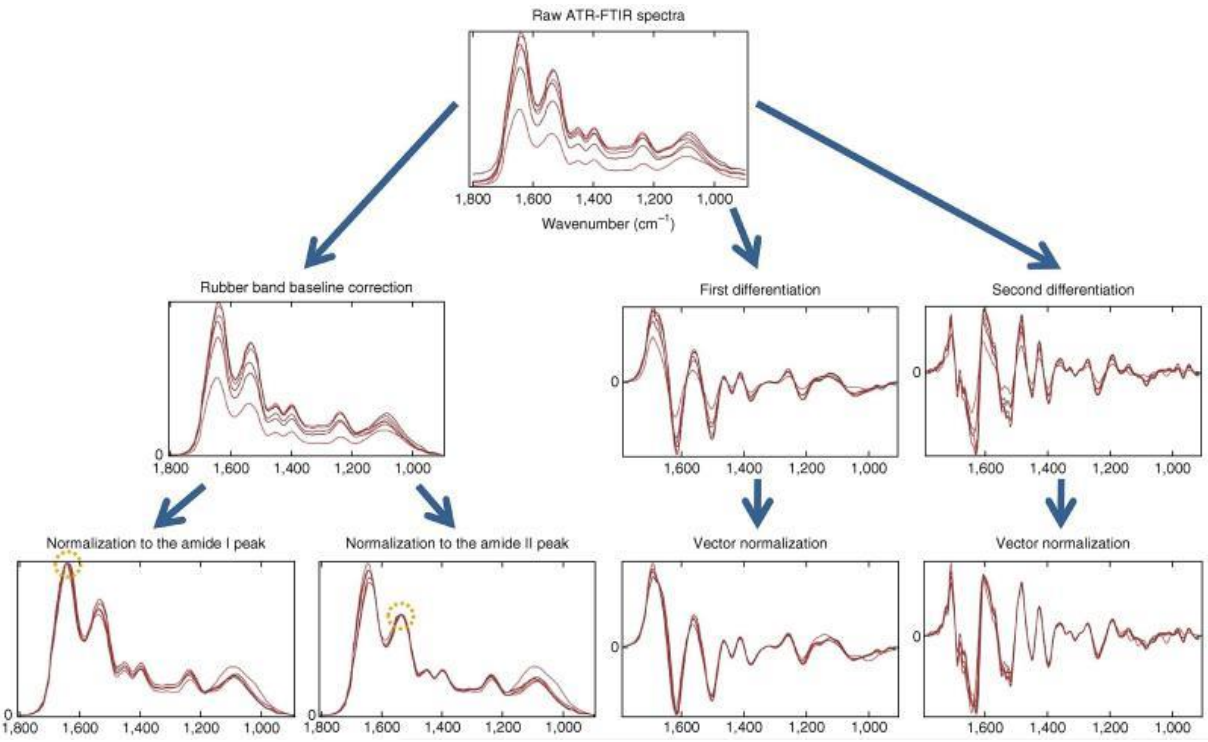

Figure 1.15 Pre-processing steps for IR spectra showing several approaches to baseline correction and normalization (from Baker et al. 2014).

1.7.3.2 Multivariate Analysis

Multivariate analysis is an essential part of advanced spectroscopic studies and is required for obtaining biologically relevant information. This is because differences between biological samples can be very distinct or extremely subtle. Moreover, when differences are plenty but are distributed across all samples, the effects may mask more indistinguishable differences between specific sample classes of interest. In order to explore the differences detected by vibrational spectroscopy, namely those present across the whole dataset, or those specific to a sample class, multivariate analysis is compulsory. Multivariate analysis is therefore a powerful tool to explore spectral datasets for biological applications (Kelly et al. 2011; Morais et al. 2017; Trevisan et al 2012).

1.7.3.2.1 Principal Component Analysis

Principal component analysis (PCA) has been one of the most effective techniques for the analysis of spectrochemical data. As a staple multivariate approach, PCA explains the variance within the highly dimensional original spectral dataset. Because it is not practical to compare all variables of the original spectrum, PCA reduces the dimensionally of the original spectrum, while retaining almost all the dataset variance (Jolliffe 2011; Trevisan et al. 2012). This simplifies the comparative variance between spectra in relation to the original wavenumbers. Through orthogonal transformation of spectra, PCA forms new axes, principal components (PCs) that best describe the dataset variance also known as the covariance (Jolliffe 2011). Reducing the complex absorbance-wavenumber spectrum into a single point within the newly generated n-dimensional discriminant space, allows for data visualization and pattern recognition (Trevisan et al. 2012). Part of this analysis strategy generates the covariance matrix, which describes the original data as scores (eigenvalues) along the individual PCs (eigenvectors). Each PC is an eigenvector offering a unique visualization of the data within the n-dimensional space. Scores provide a numerical variance value along a given PC, where each PC is orthogonal to one another and explains less variance than the previous PC (Jolliffe 2011). Figure 1.16 shows a 3-dimensional PCA space with orthogonal axes. These 3 PCs are effective at discriminating spectra of healthy corn (green) from infected corn (blue and red), into distinct clusters. This data example was generated using a handheld Raman device (Farber and Kurouski 2018).

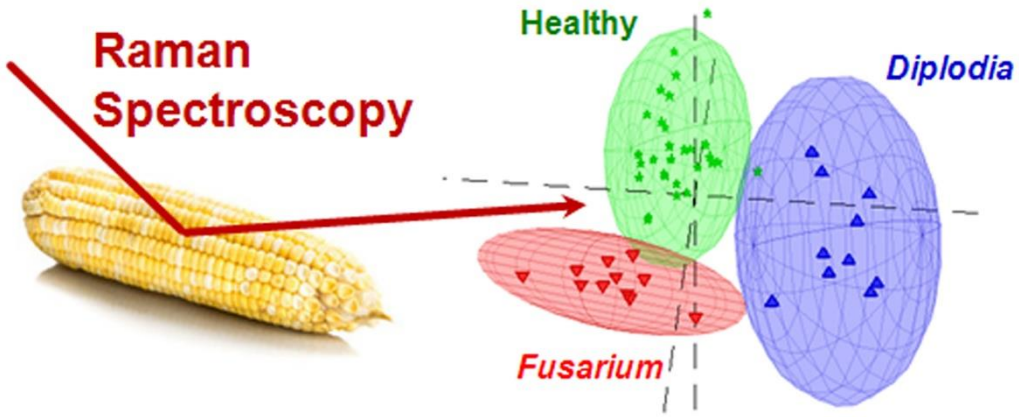

Figure 1.16 PCA illustrated on a set of infected corn (blue/red) across their control (green) showing the first three orthogonal PCs in a 3-dimensional space (from Farber and Kurouski 2018).

As an unsupervised method, PCA provides an overview of general dataset variance where class labels do not influence the analysis. Once applied the overall dataset is captured and spectra are explained by variance scores along the generated PCs. This permits further computational analysis while greatly reducing the dimensionality of the original data. Typically, the number of PCs included in subsequent analysis is chosen so that 99% or more of the dataset variance is captured (Kelly et al. 2011; Martin et al. 2010; Trevisan et al. 2012) While this is usually achieved by including the first 10 PCs, this should be examined prior to further analysis steps. To visualize PCA results, either scores plots and or loadings plots are used. PCA allows the variance within the dataset to be visualized in the form of either scatter or loadings plots. Simply put PCs are depicted in two or three dimensions, where point spectra that are closely clustered are more similar than separated ones; in other words, similar scores suggest homogeneity, while dissimilar scores represent heterogeneity (Martin et al. 2010).

1.7.3.2.2 Linear Discriminant Analysis

In contrast to PCA, LDA is a supervised method and extracts class specific differences. If materials are similar and the differences between them is subtle or masked by natural heterogeneity between samples, supervised methods such as LDA can assist in discerning these differences. Unlike PCA, which does not account for class labels, LDA in contrast minimizes intra-class differences and maximizes differences between classes, effectively emphasizing class specific separation (Kelly et al. 2011; Trevisan et al. 2012). To improve the effectiveness of LDA for investigating class specific differences, PCA is coupled with LDA to form PCA-LDA. Figure 1.17 shows PCA-LDA, where better class specific separation is observed when PCA is combined with LDA. Using PCs as input for LDA reduces the number of variables and prevents overfitting thereby improving the class specific separation. As previously discussed in relation to PCA (see Section 1.7.3.2.1), an adequate selection of PCs should be introduced into LDA, where ideally the number of PCs included contain 99% of dataset variance or more. Not including enough PCs can result in a loss of variance and thus important dataset information. Including excess PCs may lead to the inclusion of noise and lead to overfitting (Trevisan et al. 2012).

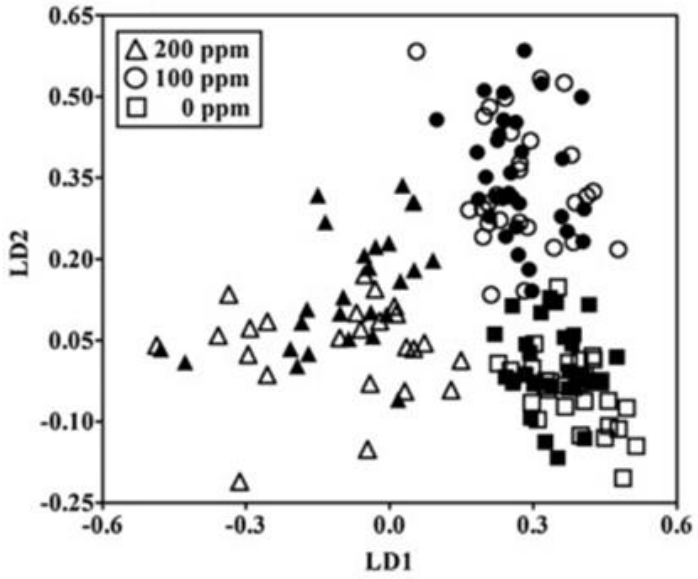

Figure 1.17 PCA-LDA discriminating guard cells (black) and epidermal cells (white) under Calcium sufficient and deficient conditions; 2-dimensional scores plot is shown (from Butler et al. 2017).

1.7.3.2.3 Classification Algorithms

Classification algorithms are useful to determine the diagnostic potential of spectral information. For decision making purposes relying on spectral data, classification algorithms provide quantitative output to assess the performance of the approach. Specifically identifying normal form abnormal states such as healthy from diseased tissues, cells, and fluids has been achieved, often with high accuracy in many biological systems. Table 1.2 shows classification results for identifying healthy and diseased corn samples autonomously from IR spectra (see also Figure 1.16). Under certain circumstances it is necessary to derive sensitivity and specificity values to determine the appropriateness of these methods (Gajjar et al. 2013; Morais et al. 2017). This is particularly true in the field of biomedicine, where samples are derived from, and influence, human subjects.

Table 1.2 Classification results for autonomous decision-making to detect disease on corn using IR spectra (from Farber and Kurouski 2018).

Class	Correct Classification (%)
Healthy	100
Diplodia	100
Fusarium	100

There are many classification models available for evaluation and it is noteworthy that approaches to spectral data analysis have not been standardized making it potentially difficult to compare methodologies. Nevertheless, the potential for automated systems, combining spectral data with classification algorithms for autonomous decision-making, to outperform subjective human evaluation has been demonstrated (Gajjar et al. 2013). Although there are numerous classification models, among the most commonly used classifiers are linear classifiers, Bayesian networks, artificial neural network (ANN), support vector machine (SVM), hierarchical cluster analysis (HCA), genetic algorithms (GA), and linear discriminant classifier (LDC) (Trevisan et al. 2012, 2014).

The primary aim of such models is for machine learning, where a computer effectively performs a specified task without explicit instructions. Attention should be given to adequate training, testing, and validation of the classification model (Morais et al. 2017; Trevisan et al. 2012). Further influencing model selection and data analysis strategy is the sample size, where both biological replicates as well as the number of spectra collected from each sample need to be considered (Beleites et al. 2008; Trevisan et al. 2014). Complementary to PCA and LDA, which have been very effective for the analysis of patterns in spectral data, is the LDC; this approach benefits from little parameter optimization by fitting a classification model to the dataset (Baker et al. 2014; Trevisan et al. 2012). A further yet more complicated classification model is SVM, which has also been used extensively for spectral datasets in the context of biological systems. Concisely, SVM generates a ‘hyperplane’, which optimally separates ‘support vectors’ that represent sections of the dataset (Morais et al. 2017). This model can be used for linear and non-linear datasets and can be used if less sophisticated models such as LDC perform poorly or overfit the model leading to failed fitting of additional data or future observations. While possibly superior

under certain circumstances, SVM poses a higher computational burden through more involved parameter selection. Regardless of the data model used, if it can be trained, tested, and validated then it may be incorporated into machine learning systems that rely on spectral input.

1.7.4 Biospectroscopy in Plant Research

The application of vibrational spectroscopy in biology is extensive and includes examination of plant materials for compound detection and quantification to *in vivo* analysis of dynamic processes (Skolik et al 2018b). However, to date, plant-based applications have not come close to the impressive strides made by the biomedical community in the quest to conquer disease through detection, quantification, and the development of practical real-world solutions to clinical management (Martin 2018). Yet for plant sciences, the versatile methods offered by vibrational biospectroscopy, specifically for the investigation of plant spectropathology, have not been fully taken advantage of. This leaves a large opportunity for knowledge exchange between biomedical and plant sciences with shared goals concerning disease research. Further, the biospectroscopy approach fits well with aspects of IPM and precision crop protection. To this end, more progress has been made in recent years as the value of plant-based biospectroscopy is becoming realized (see Chapter 2) (Butler et al. 2015, 2017; Egging et al. 2018; Farber and Kurouski 2018; Skolik et al. 2018a; Yeturu et al. 2016).

1.8 PhD Project Aims and Objectives

The principal aim of this PhD research is the advancement of MIR spectroscopy as a bioanalytical sensor technology for application in the plant and crop sciences. More specifically, the general appropriateness of IR spectroscopy as a candidate sustainable, non-destructive, fast, and precise tool for *in vivo* analysis of crop plants pre and post-harvest will be assessed. Whether the *in vivo* analysis capability can be used for baseline characterization of healthy crops and or the detection of PPs, especially at early stages will be a primary focus.

Initially the potential of biospectroscopy for general use in the plant and crop sciences will be evaluated. This will include a review of past and current applications, as well as challenges and limitations that need to be overcome to move biospectroscopy further into the agricultural sectors (Chapter 2). Recommendations are made on practical steps to using biospectroscopy as an applied sensor, by eliminating or minimizing sample preparation and using portable equipment thereby

testing if analysis of intact plants and crops is routinely possible. Spectro-chemical alterations correlating with known biological processes occurring in primarily epidermal tissue layers will be investigated, which are the primary IR targets of intact crops.

Limited research on intact plant systems to date using biospectroscopy, presents the opportunity for spectral baseline characterization for healthy development (Chapter 3) and disease research in the model system *S. lycopersicum*, of which plants and fruit were chosen as the primary model system for this PhD research. Monitoring and identification of various stages of healthy growth and development using IR spectroscopy affords the initial steps towards analysis of plant-pathogen systems found in realistic situations.

Early detection of plant-pathogen interactions at the pre-symptomatic stage is a major objective to maximize the use of current, or development of alternative, crop protection measures. These objectives will be investigated in pre and post-harvest scenarios on plants and fruit to address application potential at various points along the food production/supply chains. Damage as a pre-symptomatic indicator, and inducer of microbial infection, at the post-harvest stage will be used as a case study to determine the feasibility of pathogen detection in marketable ripe tomato crops (Chapter 4). By demonstrating the capacity of pathogen detection indirectly (pre-symptomatic) and directly (symptomatic) through the generation of spectral biomarkers, tentative disease specificity will be established for individual pathogens. Automatic pathogen detection will be evaluated using autonomous machine learning algorithms, which may in conjunction with the retooling of portable IR instruments, be readily adapted as prototype field sensors.

Fast acting microscopic pathogens that have the potential to rapidly destroy large amounts of crops are specifically detrimental to pest and pathogen induced crop loss. These pathogens are typically invisible until apparent through visible symptomatic, at which point adverse effects are certain; therefore, the chosen model organism tomato will be studied during its interaction with *Botrytis cinerea*, a model fungus at early and late stages of infection (Chapter 5).

Effects of pathogens on plant growth and development and thus crop yield is a concern for farmers and growers. IR spectroscopy will therefore be applied to the qualitative and quantitative assessment of disease associated spectral alterations in their biological context.

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Chapter 2: Biospectroscopy for Plant and Crop Science

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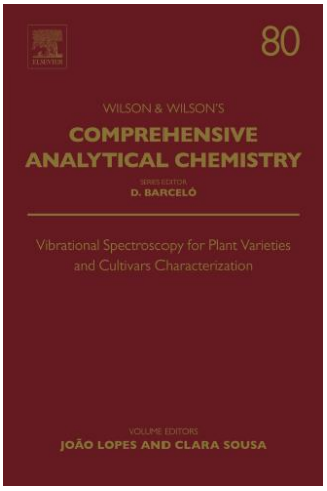

Abstract

Plants as our most renewable natural resource are indispensable within earth's biosphere, especially for food security. Providing food security in a modern world requires an ever-increasing understanding of how plants, and their products, respond to changes in the environment. In this respect, a combination of physical and chemical analytical methods can be used to study the structure and function of plants at the whole-plant, organ, tissue, cellular, and biochemical levels. Vibrational spectroscopy in biology, sometimes known as biospectroscopy, encompasses a number of techniques, among them mid-infrared and Raman spectroscopy. These techniques are well-established label-free, non-destructive, and environmentally friendly analytical methods that generate a spectral "signature" of samples using mid-infrared radiation. The resultant wavenumber spectrum containing hundreds of variables as unique as a biochemical "fingerprint" represents the biomolecules (proteins, lipids, carbohydrates, nucleic acids) present within a sample, which may serve as spectral "biomarkers" for the discrimination of distinct as well as closely related biomaterials, for various applications. In plants, biospectroscopy has been used to characterize surface structures in intact plant tissues such as leaves and fruit, plant cuticles, and cell walls, as well as to study the effects of stress on plant species. Not only does this allow the effective discrimination and "chemo-identification" of different plant structures, varieties, and cultivars, it also permits chemical profiling of plant tissues for physiological applications such as plant health monitoring and disease detection. Technical advancements are starting to overcome the major limitations of biospectroscopy such as detection sensitivity, penetration/imaging depth, and computational analysis speed, expanding the application of biospectroscopy in the plant and crop sciences. Vibrational spectra thereby serve as a basis for localization, identification, quantification of key compounds within plants, as well as to track dynamic processes for molecular-level analytics and diagnostics. This provides development potential as sensors in automatic decision-making platforms for areas including precision farming and the food production/supply chain. In this chapter we will discuss the application of biospectroscopy to study plant and crop biology and consider the potential for advancements to make biospectroscopy a more prominent technology for fundamental plant research and applied crop science as part of solutions to agricultural challenges both now and in the future.

2.1 Introduction

Plants as primary producers constitute one of the most important natural resources on earth, contributing to food security, medicine, energy, and providing a source of a tremendous amount of materials and compounds. Yet successful cultivation of plants and distribution of their products, specifically for food security, remains a key challenge in the 21st century. While malnutrition continues to plague up to one in three humans (IFPRI 2017), environmental and social issues, such as climate change, declining natural resources, harmful commercial chemicals, and population growth, confound food production. Current models of population rise predict the global total to reach between approximately 9 and 12 billion people by the end of the century (Gerland et al. 2014). The predicted global population rise merits increases between 100% and 110% for crop production by the year 2050, prompting an increase in agricultural output by approximately 60%–110% by the same year (Ray et al. 2013). However, production of at least several major crop species, including maize, corn, soybeans, potatoes, is not increasing as necessary to meet predicted future demands, and it is likely that global food demand will not be met throughout this century, especially in developing nations (Table 2.1) (Godfray et al. 2010; Mahlein 2016; Ray et al. 2013). Besides direct economic value, crop losses additionally contribute to subsequent losses for, or impacts on, consumers, health systems, global resources, and the environment (Savary et al. 2012). Not only does the insufficient production of staple crops contribute to the problem of attaining food security, waste is also a major factor throughout the food production and food supply chains (Godfray et al. 2010). Pre- and postharvest crop loss contributes to the 40% of all food lost to waste in both developed and developing nations (Godfray et al. 2010). This level of loss and waste throughout the food system seems removed from a precision and sustainable agricultural framework.

Table 2.1 Predicted annual crop production and deficits until 2050 in major crops of Maize, Rice, Wheat, and Soybean (adapted from Ray et al. 2013).

Crop	Estimated Annual Production (%)	Estimated Annual Deficit (%)
Maize	67	33
Rice	42	58
Wheat	38	62
Soybean	55	45

Plant disease is one of the major threats to food security and crop cultivation. Unfavorable environmental conditions, pests, and pathogens can destroy whole crops or reduce the quality of plant products in both pre- and postharvest situations (Strange and Scott 2005). Nonbiological factors negatively affecting plants and crops induce abiotic stress, while biological threats illicit biotic stress responses, which if overcome lead to plant disease (Bostock et al. 2014). Biotic and abiotic stresses, alone or in combination, negatively influence plant physiology, harming growth, and development, leading to reduced crop yields (Suzuki et al. 2014). Especially stress combinations can have synergistic effects, leading to more crop loss than the sum of individual stresses (Figure 2.1) (Suzuki et al. 2014). This is especially true with combinations of abiotic stresses and abiotic–biotic stress combinations (Suzuki et al. 2014). Additionally, reduced crop yields (losses) due to pests and pathogens remain significant determinants in effectively increasing horticultural production (Oerke and Dehne 2004). Pest and pathogen induced disease can reduce global annual yield by 40% for major agricultural crops (Oerke 2006), while similarly, postharvest, pathogens can infect produce during transport, storage, and household consumption leading to regular losses over 35% (Godfray et al. 2010; Oerke 2006). Such levels of crop loss and food waste suggest that current methods of crop protection and postharvest handling of horticultural goods are still inadequate under a modern precision farming framework. Part of the reason is the lack of commercial analytical and diagnostic tools to detect threats to plants and crops throughout the food production and food supply chains (Mahlein 2016).

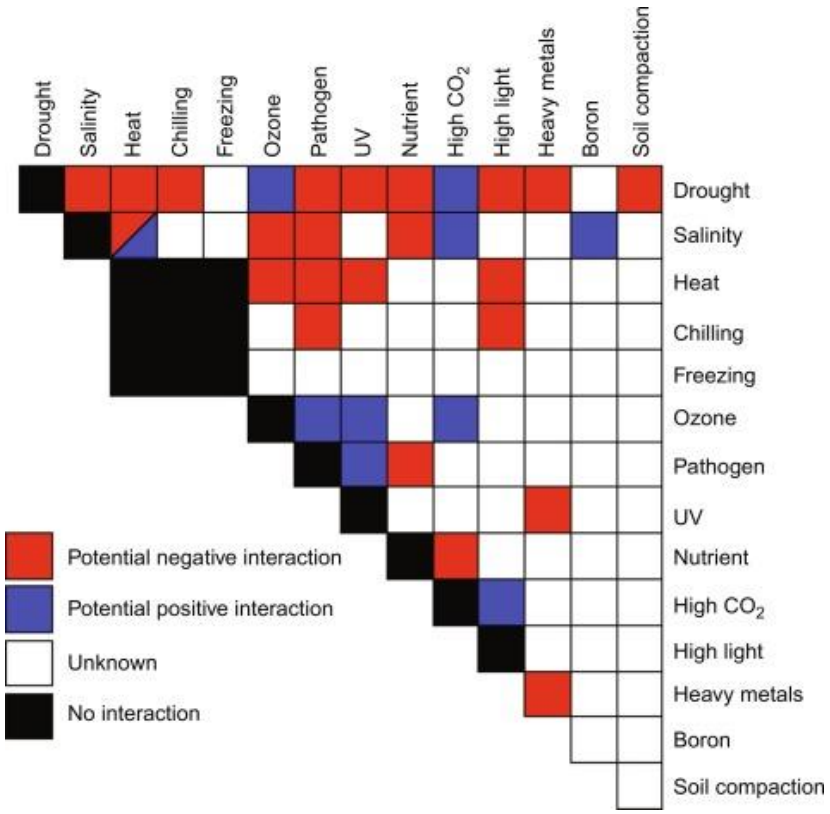

Figure 2.1 Stress interaction matrix showing stress combinations (abiotic/biotic) including synergistic (positive) and antagonistic (negative) interactions among others (adapted from Suzuki, et al. 2014).

The complexity of plants and their interactions with the environment, including biotic and abiotic factors, are therefore prominent research areas requiring analytical and diagnostic tools for fundamental plant biology and crop science, in efforts to develop solutions to horticultural losses. An increased physiological and molecular-level understanding of how plants respond to changes in the environment (biotic and abiotic factors) and how these conditions influence the composition of valuable plant substances for human use would therefore be beneficial for making improvements in horticulture. For this, a combination of physical and chemical analytical methods is available to study static and dynamic structure–function relationships of plants at the whole-plant, organ, tissue, cellular, and biochemical level. These include both destructive and nondestructive methods, such as nucleic acid-based or traditional molecular techniques that are destructive, as well as proximal and distal optical sensors such as spectroscopy, remote sensing,

and volatile organic compound (VOC) analysis, which are possible nondestructively (Martinelli et al. 2015). Combined, these sensor-based methods share common goals aimed at the following priorities: plant health monitoring and pre-symptomatic disease detection; identification of different plant varieties and cultivars including plants naturally resistant to stress for genotyping/phenotyping and taxonomic classification purposes; and increasing mechanistic insight into plant physiology and disease (Mahlein 2016; Martinelli et al. 2015). Nondestructive optical sensor technologies have gained popularity due to the possibility to study plants in their natural context, while gaining biologically important information relevant to fundamental plant research and applied crop science (Mahlein 2016). Many of these technologies can therefore contribute simultaneously to laboratory based as well as industry applied efforts toward crop protection. To this end, various nondestructive optical sensors have shown promise, in the context of crop loss and food waste, by contributing knowledge to both fundamental plant research and applied crop sciences (Mahlein 2016; Martinelli et al. 2015; Sankaran et al. 2010).

Mid-infrared (MIR) vibrational spectroscopy of biological materials has developed into a versatile tool for fundamental plant research, with development potential as a sensor for applied crop sciences. Vibrational spectroscopy in biology known as biospectroscopy encompasses well-established label-free, nondestructive, and environmentally friendly analytical methods that generate wavenumber spectra of samples using MIR radiation. A wavenumber spectrum contains hundreds of variables as unique as a biochemical "fingerprint," and reflects the biomolecules (proteins, lipids, carbohydrates, and nucleic acids) present within a sample (Baker et al. 2014). Changes in specific wavenumber variables of the IR spectrum may serve as spectral "biomarkers" for the discrimination of distinct as well as closely related biomaterials, for various applications (Martin et al. 2010). Among the most common biospectroscopy methods are MIR and Raman spectroscopy. General applications of biospectroscopy include the study of biomolecules *in vivo*, cell variability and identification of particular phenotypes, and measuring biochemical processes on the cellular and subcellular level both spatially and temporally (Quaroni and Zlateva 2011). Measuring concentration gradients, the orientation of biomolecules in living cells, distinguishing cellular phenotypes, detection of specific metabolites or compounds, as well as measuring dynamic changes in biomolecule abundance and distribution are routine applications of biospectroscopy (Martin et al. 2010; Quaroni and Zlateva 2011). Plant and crop science specific application highlights of biospectroscopy include the characterization of many valuable plant substances such

as primary and secondary metabolites; investigation of plant surface structures including external barriers like the cell wall and cuticle; chemo-identification plant varieties and cultivars; as well as measuring effects of major abiotic and biotic stress conditions (Heredia-Guerrero et al. 2014; Largo-Gosens et al. 2014; Ord et al. 2016; Schulz and Baranska 2007; Zimmermann et al. 2015). This includes studies on various plant parts including samples from leaves, fruit, roots, stems, and pollen, highlighting its applicability to various plant systems (Fu et al., 2016; Ribeiro da Luz, 2006; Stewart et al. 1997; White et al. 2016; Zimmermann et al. 2015). Although not widely used to date, the generally nondestructive and reagent free sample preparation toward biospectroscopy analyses potentially facilitate a wide range of applications that have been underexplored. In order to develop biospectroscopy further, specifically toward being an applied sensor for industry and laboratory alike, more non-destructive *in vivo* investigations using biospectroscopy are needed (Butler et al. 2015). While the number of *in vivo* studies of biospectroscopy is to date limited primarily by technical limitations, sufficient proof of concept exists to warrant further evaluation for intact plant and crop analysis without destructive sample preparation as a prerequisite. To this end, recent developments show that spectral alterations of measurements taken *in vivo* within intact plants and crops can be physiologically representative (Butler et al. 2015; Fu et al. 2016; Trebolazabala et al. 2013). Further, semi- and fully portable MIR and Raman spectrometers are available for potential field use. Several recent studies have investigated semi-portable and miniature biospectroscopy equipment, primarily Raman, for *in vivo* measurements in tomato fruit (Fu et al. 2016; Trebolazabala et al. 2013). Applications of biospectroscopy to date in the laboratory and industry, combined with recent developments and the increasing availability of portable biospectroscopy systems, suggest that these methods may become rapidly adapted to serve as sensors for field applications in various pre- and post-harvest scenarios. Because biospectroscopy is inherently inter-disciplinary, research collaborations, knowledge transfer, together with technical advancements and the evaluation of fully portable biospectroscopy equipment, will contribute to biospectroscopy sensor development, facilitating better crop protection and reducing food waste in future food production/supply. Herein, we summarize the main concepts of biospectroscopy including sample preparation, spectral acquisition, and data analysis of MIR and Raman spectroscopy. Biospectroscopy applications to valuable plant substances, select surface structures such as the cuticle and cell wall, identification of varieties and cultivars, as well as plant–environment interactions, including abiotic and biotic stress, are reviewed. Analysis of crop plants,

intact specimens (*in vivo* or *in situ*), as well as the use of more portable systems is highlighted. We also outline select challenges and limitations specifically relevant to the transition from lab instrument to field sensor. Further, we briefly suggest novel directions for biospectroscopy in plant and crop sciences.

2.2 Biospectroscopy

Biospectroscopy refers to a collection of techniques including, but not limited to, MIR and Raman spectroscopy for applications in biology. These techniques rely on the interaction between infrared (IR) radiation and the functional groups present in biomolecules, to generate a unique IR spectrum over the range of wavelengths from 2.5 to 25 μm, converted to energy units in wavenumbers (4000–400cm^{-1}). Energy in the IR range causes molecular excitation, vibration, and rotation of molecules within a biological sample. Functional groups present in biomolecules, such as proteins, lipids, carbohydrates, and nucleic acids, characteristically interact with IR radiation (Baker et al. 2014). Biochemically complex samples such as those from biological materials therefore produce information rich and highly characteristic spectra for multicomponent analysis (Moros et al. 2010). The highly characteristic and unique IR spectra also referred to as samples biological "fingerprint" spectrum or IR "signature" are exceptionally useful to distinguish between remarkably similar samples based on minute biochemical alterations. IR spectra therefore provide the basis for classification and characterization, as well as tracking both large and small biochemical changes over time (Quaroni and Zlateva 2011). Distinct light–matter interactions are measured by MIR and Raman spectroscopy over the same energy range (4000–400 cm^{-1}), producing unique but complementary information, and when used in combination provide a more detailed analysis of the sample. Various sample modes allow the probing of different sample areas covering spatial resolution from the nanometer scale up to spectra representing areas covering square millimeters (Kazarian and Chan 2013), which permits the interrogation of biological systems at various levels of biological organization.

2.2.1 Mid-Infrared Spectroscopy

MIR spectroscopy relies on light absorption. Incident IR light upon a sample causes biochemical bonds to vibrate. Vibrational modes of molecule thereby cause specific amounts of energy from the incident IR beam to be absorbed, reducing the intensity of the subsequently

detected IR beam. The difference in energy between incident and detected IR radiation produces a complex interferogram, which is deconvoluted using a FT operator (Stuart 2004). This separates the individual wavelengths of the measured IR range into component wavelengths, producing a wavenumber spectrum. MIR spectroscopy, in contrast to Raman spectroscopy, relies on a dipole moment present only in diatomic or more complex molecules, which is often not a limitation within biological materials.

2.2.2 Raman Spectroscopy

Raman spectroscopy relies on molecular excitation by way of polarization and subsequent light scattering. Incident photons from the IR laser source interact with a molecular configuration resulting in elastic or inelastic light scattering. Elastic light scattering, known as Rayleigh scattering, predominates resulting in no net energy transfer between incident IR radiation and sample molecules, providing no information and therefore filtered out (Smith and Dent 2013). Alternatively, a net energy decreases or increase in the scattered IR light results in inelastic light scattering known as Stokes Raman and anti-Stokes Raman scattering, respectively (Andrews 2014). Fluorescence can interfere with the detector signal due to its occurrence at similar energy transitions as those detected by Raman spectroscopy. Raman scattering measured over the same energy range as MIR spectroscopy (4000–400 cm^{-1}) but each measuring distinct light–matter interaction phenomena, makes these two methods complementary. Various forms of Raman spectroscopy make use of these phenomena in different ways, expanding the application potential of these techniques to biological analysis (Butler et al. 2016).

For a detailed account of MIR and Raman spectroscopy theory, which is out of the context of this discussion, more information can be found in the literature (Andrews 2014; Baker et al. 2016a; Smith and Dent 2013; Stuart 2004). Because of its versatility, it is important to make the appropriate choices at each stage of the biospectroscopy process to meet the intended research aims and objectives. To aid experimental design and provide an overview, the biospectroscopy method may be divided into three component parts including sample preparation, spectral acquisition, and computational analysis (Kelly et al. 2011b; Trevisan et al. 2012). A number of protocols have become available for new users with guidance on sample preparation, spectral acquisition, and computational analysis for a number of samples. These protocols exist for both

MIR and Raman spectral analysis of biological materials (Baker et al. 2014; Butler et al. 2016; Gierlinger et al. 2012).

2.2.3 Sample Preparation

Vibrational spectroscopy can measure virtually any type of organic material; however, sample preparation may considerably alter the vibrational spectrum compared to the sample in its native state. Many types of sample preparation are employed within the plant laboratory including physical and or chemical modifications of the sample. Concerning plants, common sample preparation may include cutting, drying, grinding, homogenization, fixation, fractionation, purification, etc., most of which influence the IR spectrum to some degree. Benefits of sample preparation include the ability to take complex biological systems including plant cells and tissues and separate them into less complex constituents for a more precise characterization of individual substances. Several studies have investigated select effects of sample preparation on IR spectra under specific conditions (Bureau et al. 2012; Zohdi et al. 2015). Nevertheless, extensive sample preparation consumes time and resources while altering the native architecture of biological tissues, which may limit or remove any physiological context of the resultant data, and thus there is a general desire to perform analysis *in vivo* where possible. Despite the ability to measure a vast array of sample types, the use of biospectroscopy for *in vivo* measurements has been surprisingly limited, especially in the plant and crop sciences. Nonetheless, biospectroscopy is being applied to an increasing amount of *in vivo* systems, and a number of studies have demonstrated that *in vivo* analysis of whole cells and tissues is readily achieved (Butler et al. 2015; Fu et al. 2016; Heraud et al. 2005; Trebolazabala et al. 2013). These studies demonstrate that analysis of both processed and native samples is possible, and while different types of sample preparation precede most biospectroscopy studies to date, the continued development of *in vivo* analysis is favorable for the nondestructive measurements of physiologically active plants and crops (Butler et al. 2015). Additionally, the analysis of plant cells and tissues *in vivo* requires practically no sample preparation, while having the benefit of being physiologically representative under native conditions, which contributes to faster spectral acquisition, increasing the relevance for future industry applications.

2.2.4 Spectral Acquisition

Instrument choices influence the area of interrogation, sensitivity, spatial resolution, and acquisition speed of measurements in biospectroscopy. Three main sampling modes are available for biospectroscopy; these are transmission, reflectance, and attenuated total reflection (ATR) spectroscopy (Smith 2011). These sampling modes are applicable to both MIR and Raman spectroscopy. For MIR in transmission mode, light passes through the sample and, due to the energy of MIR, is generally limited to thicknesses up to 20 μm (Smith 2011). Furthermore, because most intact biological samples are thicker than this limit, sample preparation is necessary, although this has beneficial effects on the quality of resultant spectra (Butler et al. 2017). Thus, for *in vivo* analysis, transmission mode may be limited to specific samples suitable to this acquisition mode. In contrast, Raman transmission spectroscopy has a sample thickness capacity in the range of 30 mm, expanding this sampling mode to thicker plant tissues (Butler et al. 2016). This is because the laser sources used for Raman analysis generally have higher energy than regular MIR excitation sources and thus penetrate deeper into biological samples. The same is true for traditional Raman scattering, where laser light penetration into plant tissues is in the range of several hundred micrometers (Butler et al. 2015). For Raman spectroscopy, additional light–matter phenomena may be exploited through a number of adaptions of the technique including surface enhanced Raman spectroscopy (SERS) and stimulated Raman scattering applied to plant samples (Butler et al. 2016; Littlejohn et al. 2015; Zhang et al. 2017). Although not common outside specialized applications, MIR spectroscopy utilizing synchrotron radiation from specialized particle accelerators can provide an exceptionally bright excitation source for spectral acquisition in several specialized adaptions of biospectroscopy to plants and pathogens (Butler et al. 2017; Holman et al. 2010; Kaminskyj et al. 2008).

Sensitivity and selectivity of MIR and Raman spectroscopy are dependent on method and instrument choice. Sensitivity is the detection limit for a particular chromophore, as determined by its absorption relative to background noise, while the selectivity is the capability of detecting specific chromophores within a mixture (Quaroni and Zlateva 2011). Sensitivity and specificity parameters may be optimized depending on the aim of the experiment and are important to determine if biospectroscopy techniques are suitable for the study, compared to other available analytical methods. Spatial resolution covers macroscopic (macro-measurements) areas down to nanometer level resolution (micro-measurements) depending on the method used (Baker et al.

2016a; Kazarian and Chan 2013). While a significant amount of effort is put into improving spatial resolution on the micro and nanometer levels, macroscopic measurements covering several square millimeters or centimeters may be more appropriate for measuring physiological processes in whole plant organs, especially for rapid routine analysis. Although micro-measurements permit the imaging or mapping of specific regions at cellular and subcellular resolution.

Spectral resolution refers to the number of variables generated in a spectroscopic measurement. A spectral resolution of 4 cm^{-1} would generate roughly twice as many wavenumber variables in the spectrum as a spectral resolution of 8 cm^{-1}, thus increasing scan time significantly (Quaroni and Zlateva 2011). Depending on instrument choice, and measurement area, spectral acquisition time will vary significantly. It is possible to choose sampling modes, which allow the optimization of measurement area, penetration depth into the sample surface, as well as spatial and spectral resolution, all of which have an impact on spectral quality and acquisition speed (Quaroni and Zlateva 2011).

2.2.5 Computational Analysis

Extracting wavenumber variables from biological samples to serve as "spectral biomarkers" related to a specific effect or treatment requires computational analysis in order to answer biologically relevant questions. IR and Raman spectra contain hundreds of variables with both qualitative and quantitative attributes for analysis (Baker et al. 2014). In general, extracting biological information from vibrational spectroscopy data falls into two main categories consisting of exploratory and diagnostic frameworks (Trevisan et al. 2012). The exploratory framework focuses on data visualization and direct comparisons of spectral groups for primarily qualitative analysis and characterization of spectral features (Trevisan et al. 2012). A more involved approach, following or combined with an exploratory framework, is the diagnostic framework. This approach requires extensive design, validated spectral datasets, combined with machine learning based on quantitative features, with the goal of autonomous classification of spectra from specific classes/treatments (Trevisan et al. 2012). It should be noted that although conceptually separate, the exploratory framework almost always precedes or is used in conjunction with the diagnostic framework, as the development of diagnostic frameworks requires validation (discussed later) (Trevisan et al. 2012). Common to both frameworks are processing steps including preprocessing, normalization, and computational analysis. Preprocessing and normalization are necessary to make

spectra comparable to each other by minimizing the influence such as sample thickness and instrument variability (Baker et al. 2014; Martin et al. 2010; Trevisan et al. 2012). Computational analysis as part of both exploratory and diagnostic frameworks makes use of various chemometrics including univariate, multivariate, and ratiometric analysis, which use single variable, multiple variables, or ratios of variables, respectively (Kumar et al. 2016b; Trevisan et al. 2012). These approaches extract spectral "biomarkers" (wavenumber variables) to serve as indicators of class or sample treatment (e.g., normal, abnormal, and diseased) (Kelly et al. 2011b; Martin et al. 2010). Among these variables, extracting relevant ones consistent with sample treatment, rather than naturally occurring variance as is the often the case with biological samples, can be difficult. In cases where high naturally occurring heterogeneity exists, a combination of unsupervised data reduction steps combined with supervised methods focusing on the inter-sample differences has been effective. Among others, principal component analysis (PCA) and linear discriminant analysis (LDA) have been efficient at providing insight into natural population heterogeneity and class-specific differences, respectively (Martin et al. 2007, 2010), as part of exploratory inquiry. Classifier algorithms including linear discriminant classifier or support vector machines (SVM) are commonly used for biospectral datasets as part of diagnostic frameworks (Trevisan et al. 2012). A large number of analysis models are available many of which can be combined to form composite techniques such as PCA–LDA or PCA–SVM (Trevisan et al. 2012). Ultimately, the exact data analysis options are dependent on the questions set out to answer, in addition to the goals of individual research groups. Further details pertaining to computational analysis of biospectroscopy data, and considerations for exploratory and diagnostic frameworks, can be found elsewhere (Kelly et al. 2011b; Trevisan et al. 2012). Combined, the exploratory and diagnostic frameworks offer insight into the mechanistic biology of the study, while the diagnostic framework evaluates the classification accuracy of spectra belonging to specific sample classes. If the accuracy of a diagnostic framework is sufficiently high, it may warrant evaluation in automated decision-making platforms for subsequent use in high-throughput systems for commercial applications (Stables et al. 2017).

As part of an effort to increase the biological relevance through mechanistic insight into molecular changes relating to spectral biomarkers, on which diagnostic and exploratory frameworks rely, catalogues of spectral markers for both MIR and Raman spectroscopy are available. These catalogues, originally published in 2007 for Raman and 2008 for MIR, are aimed

at providing a guide for the interpretation of spectral bands, with recent updates to these spectral catalogues reflecting the increasing use of these spectroscopies (Movasaghi et al. 2007, 2008; Talari et al. 2015, 2017). In future, these will likely become available specifically for plant materials (Heredia-Guerrero et al. 2014; Largo-Gosens et al. 2014). Biological spectra thus provide plant biologists with molecular-level information, while providing industrial horticulturalists with rapid classification systems for detecting differences in sample material, thus contributing simultaneously to laboratory and field-based applications.

2.3 Biospectroscopy for Fundamental Plant and Research and Applied Crop Science

2.3.1 Valuable Plant Substances

Preventing crop loss and improving our understanding of valuable substances for human consumption require rapid identification and characterization of plant constituents in intact plants and crops as well as processed plant products. Plants are composed of, and produce, an impressive array of organic substances for human consumption as food and plant-based bio-commodities. The quality of these substances often depends on the healthy growth and development of their respective plants, whether it is the plant itself that is the target substance, or a derivative product collected subsequently. Fruits and vegetables, medicinal compounds, phytonutrients beneficial for human health, as well as structural biopolymers are only a few examples of valuable plant substances for everyday use. In nature, primary metabolites, or core metabolites, such lipids, proteins, carbohydrates, and nucleic acids are essential for the healthy growth and development of all plant species; secondary metabolites, although not necessarily essential to survival, confer species specificity and provide specialized functions such as plant defense. Metabolites along with other classes of plant compounds thereby provide natural markers to study developmental and physiological processes in plants. Further, metabolites or other biochemical targets are quality indicators in horticultural processes and the food industry. Various primary and secondary metabolites, among other plant constituent compounds, are therefore important for research and industry (Lohumi et al. 2015; Rodriguez-Saona and Allendorf 2011). This applies to valuable substances in processed materials, as well as to intact plant tissues. For a better understanding of the biochemical composition of crop plants and their products, continued identification, characterization, and quantification of the various classes of plant substances are necessary.

Especially the development of nondestructive tools to analyze delicate and potentially highly heterogeneous samples, such as fruits, vegetables, and whole plants, would facilitate improved crop cultivation and the production of valuable plant products. While the analysis of target plant substances is readily achieved in processed materials, tracking multiple plant substances simultaneously within whole tissues without destructive effects remains challenging.

Current uses demonstrate that biospectroscopy is adapted to study the abundance, distribution, and change of metabolites and other target substances in plants and crops. Biospectroscopy is specialized for measuring single or multiple compounds simultaneously, which has significantly contributed to a better understanding of plant constituents, valuable substances they produce, and how these substances change under both natural conditions and in response to physical and chemical processing. Specifically, the food industry has used the sensitivity of IR and Raman spectroscopy extensively for quality control and detecting the corruption of various food products based on select compound detection and quantification (Lohumi et al. 2015; Rodriguez-Saona and Allendorf 2011). However, most commercial food industry applications are limited to homogenous bulk samples such as oils, flours, and dairy (Karoui et al. 2010). Transferring the food analytics application to more difficult specimens, such as whole production, without any sample manipulation remains difficult, although progress is being made using a number of economically important crops. Common plant metabolites have been characterized by IR and Raman spectroscopy including primary metabolites, such as amino acids (proteins), fatty acids (lipids), and carbohydrates besides secondary metabolites including phenolics, terpenoids, alkaloids, and polyacetylenes (Baranska et al. 2013; Schulz and Baranska 2007). Biospectral analysis of primary metabolites within crop species includes the measurement of wheat protein in kernels, lysine distribution in barley, and the study of temperature and water on gluten structure under processing conditions (Georget and Belton 2006; Schulz and Baranska 2007; Thygesen et al. 2003). Lipid composition of many important plant oils has been investigated as related to the food industry (Schulz and Baranska 2007). Biospectral analysis of many common carbohydrates has been studied including the characterization of mono-, di-, and polysaccharides including cellulose (Schulz and Baranska 2007). Carbohydrates specifically have been studied in various tissues including crop species including apricot, carrot root, and onion (Baranska et al. 2013; Bureau et al. 2009). More recently, Raman spectroscopy was used to map changes in polysaccharide distribution in cell walls of apple during fruit development and senescence (Chylin

ska et al. 2016). While these present only a fraction of the literature available that pertains to primary metabolite analysis in crop species using biospectroscopy, they readily illustrate the capacity for metabolomics analysis based on primary metabolites that serve as both quality indicators and markers of dynamic biological processes.

Secondary metabolite analysis, specifically carotenoids, has gained significant attention and has been extensively studied using biospectroscopy approaches. A common goal among these studies is the development of alternatives to more traditional analytical chemistry methods such as fractionation combined with gas and high-pressure liquid chromatography, which require tissue destruction (Baranska et al. 2006b; Kumar et al. 2016a). Tomato fruits and related products such as juices have been focus points for biospectroscopy studies of plants, likely due to their nutritional value and relevance as a popular crop (Fu et al. 2016; Radu et al. 2016). Secondary metabolites associated with fruit ripening, including lycopene, β-carotene, phytoene, and phytofluene, have been subject of study using MIR and Raman spectroscopy (Baranska et al. 2006a; Fu et al. 2016; Johnson et al. 2003; Radu et al. 2016; Scibisz et al. 2011; Trebolazabala et al. 2013). Several of these studies have achieved metabolic profiling in whole tomato fruit, measuring metabolites such as lycopene, β-carotene, phytoene, and phytofluene in whole tomato fruit without sample preparation (Fu et al. 2016; Trebolazabala et al. 2013). Raman spectroscopy may be exceptionally suited for this specific application, as these studies both used semiportable systems capable of intact fruit analysis.

Metabolic profiling of valuable plant substances *in vivo* using biospectroscopy will help develop solutions for the quality control of crops and identify new targets for tracking physiological processes. Spectrochemical profiling using biospectroscopy will be especially useful in whole tissues, such as leaves and fruit, applied to physiological processes such as plant and fruit development, maturation, and decay. With this comes the potential application of biospectroscopy to contribute to molecular insight into the mechanism of plant and fruit development *in vivo* from the physiological perspective, while developing new quality control parameters for assessing delicate horticultural products. This in turn will facilitate application development for pre- and post-harvest sectors, where, for example, the real-time determination of development stage in the field may augment visual ripening scales, which remain the default standard (Mahlein 2016). As a result, better estimation of shelf life and related applications including the detection of defects in crops and produce, nondestructively and in real time, may become possible.

2.3.2 Species Identification

Valuable plant substances act as natural markers on which to identify and classify plant varieties and cultivars. As part of modern horticulture and plant research, it is desirable to identify, characterize, and classify plant varieties, cultivars, based on a number of sample types. Depending on the application, this may be from homogenous samples, such as processed products as part of food analytics, to larger specimens such as whole leaves. Because plant substances stretch over various levels of biological organization from single metabolites to whole plant organs, there are a many natural labels on which to identify species and classify them, as previously discussed, these natural labels include metabolites and other nonmetabolic plant substances. Species identification based on plant substances is readily achieved with biospectroscopy. There are many examples of species differentiation and chemical-based taxonomic classification using biospectroscopy in a diverse set of cultivars. Further, species identification has been performed mainly on leaf and fruit tissues, with the unique exception of pollen. For most taxonomic studies to date using biospectroscopy, samples from plant organs like leaves are dried and/or homogenized, suggesting the development for species identification *in vivo* under natural conditions. Among the many crops studied for species identification are mint, ginseng, olive, strawberry, and samples from various other plant species including Chinese ornamentals.

Rösch et al. (2002) used Raman spectroscopy of stem cross sections successfully to characterize related mint species (*Mentha* sp.). Very recently, SERS was successfully applied to study inter-cultivar differences between Chinese ornamental (*Chrysanthemum* sp.) for taxonomic purposes (Zhang et al. 2017). Rapid discrimination of strawberry cultivars based on homogenized fruits was also effectively performed (Kim et al. 2009). Kim et al. (2004) had also previously used MIR spectroscopy for the taxonomic discrimination of seven flower plant species based on homogenous samples. Similarly, homogenous dried leaf samples were subject to MIR spectral analysis to distinguish plant populations and the effects of temperature on spectral features (Khairudin et al. 2014). Aouidi et al. (2012) also used ground leaf tissue to study and distinguish five Tunisian olive cultivars (*Olea europaea*) with MIR spectroscopy. Ages of ginseng cultivars were also determined based on biospectroscopy data, whereas in most previous studies, samples were homogenized prior to analysis (Kwon et al. 2014). ATR-FTIR spectroscopy on dried but intact sage leaves (*Salvia officinalis*) was performed by Gudi et al. (2015) for more rapid

taxonomic classification, where intact leaves represent more conserved tissue architecture. What is interesting is that analysis on whole hydrated leaves was performed close to a decade earlier. Ribeiro da Luz (2006) used hydrated whole intact leaves from 15 different native tree species in the Washington, DC, area to assess the use of ATR-FTIR spectroscopy for classification of species. Using a spectral database, a classification accuracy of over 80% was achieved. This is particularly important as whole leaves are more representative samples compared to ground and homogenized tissue samples for the development of *in vivo* species identification. Uniquely, pollen has been used as a discriminating factor for species identification on two accounts, investigating both environmental effects and species differences in pollen from 300 plant species without sample manipulation (Zimmermann and Kohler 2014; Zimmermann et al. 2015). Taken together, these studies show that while work is needed for *in vivo* species identification using biospectroscopy, chemical-based taxonomy on a variety of samples is possible, including intact plant parts.

Biospectroscopy has contributed to method development for species identification and chemical-based taxonomy from a number of plant samples with future applications in genotyping/phenotyping. Species identification on various levels of biological organization may prove useful to expedite the current time required for conventional phenotyping, which is approximately 10 years from initial screening to available cultivar (Mahlein 2016). As biospectroscopy is sensitive enough to detect changes within individual nucleotides, as well as over macroscopic areas, there is potential for both genotyping and phenotyping applications in practice (Kelly et al. 2009, 2011a; Ribeiro da Luz 2006). However, the development of *in vivo* biospectroscopy is becoming a reality for various dynamic processes including physiological applications, which are readily transferrable to species identification and thus nondestructive phenotyping, or potentially even genotyping when combined with sample preparation (Butler et al. 2015; Kelly et al. 2009, 2011a).

2.3.3 Plant Surface Structures

The cuticle and cell wall contribute significantly to biospectroscopy measurements of intact plant tissues *in vivo*. As essential surface barriers, the cuticle and cell wall are conserved in all terrestrial plants and are intricately connected as part of the upper and lower epidermis. In their natural arrangements, the cuticle–cell wall layer consists of a complex matrix composed primarily of carbohydrates, proteins, and lipids (Domínguez et al. 2011). While the main function of the

cuticle is to prevent water loss and regulate gas exchange, it is also involved in defending against light damage and microorganism invasion (Domínguez et al. 2011). The cell wall defines cell shape and size and gives structural plasticity to plant cells; it is involved in plant growth, cellular differentiation, cell–cell communication, water regulation, and defense responses (Cosgrove 2005). Not only is the cell wall an essential barrier but also source of the most abundant natural biopolymer cellulose (Cosgrove 2005). Hence, the cell wall is both physiologically indispensable for plants and humans alike. Physiological and environmental cues influence the structure of both cuticle and cell wall, making these surface structures important subjects for plant and crop sciences. Yet the detailed biophysical properties of plant surface structures are difficult to discern, and few methods exist to effectively study the molecular complexity the cuticle and cell walls in their native arrangements where the two layers are elaborately intertwined (Domínguez et al. 2011).

Biospectroscopy has offered a unique look at both cuticle and cell wall composition individually and together. The small penetration depth of MIR radiation into biological tissues requires characterization of plant surface structures such as the cuticle and cell wall. MIR radiation used by biospectroscopy methods penetrates between a micron and several hundred micrometers into biological tissues depending on the method (Butler et al. 2015; Kazarian and Chan 2013).

2.3.4 Cuticle

MIR and Raman spectroscopy have augmented advances in our knowledge of functional groups contained in the cuticle matrix, their structural roles, as well as their macromolecular arrangement. Analysis of isolated cuticles was performed as early as (1992) by Chamel and Marechal, as well as on tomato by Ramirez et al. (1992). Subsequently several studies look at both isolated cuticles and cuticles as part of natural plant structures such as leaves and fruits. Biospectroscopy of plant cuticles encompasses many important and economically relevant crop plants such as potato, grasses, and tomato, many of which have very different natural morphologies. Dubis et al. (1999) used MIR spectroscopy in ATR mode as an analytical tool to investigate primarily carbonyl compounds associated with plant species and seasonal variation. This study included analysis of leaves from important crops including various cultivars of potato (Solanum tuberosum), beside several tree species. Dubis et al. (2001) also studied cuticle wax composition in hops (*Humulus lupulus*). Cuticle fractions from olive leaf, pepper fruit, and apple

fruit have also been investigated expanding the application to several important crop species (Johnson et al. 2007). Progress toward *in vivo* applications of biospectroscopy for cuticle structure analysis for both agrochemical research and plant science has recently been made in the model organism *Arabidopsis thaliana* and a cuticle-deficient mutant *eciferum1* (cer1) (Littlejohn et al. 2015). Much of this work has led to under- standing not only cuticle composition and constituent distribution, but also how cuticles change in response to exogenous factors, and during growth and development. To this end, spectroscopic characterization of plant cuticles and constituents, including cutin, cutan, waxes, polysaccharides, and phenolics, has recently been reviewed in detail by Heredia-Guerrero et al. (2014).

2.3.5 Cell Wall

Similar to plant cuticles, MIR spectroscopic analysis of plant cell walls was described over 25 years ago, likely owed to its significance in plant biology and industry (Cosgrove 2005; McCann et al. 1992). In 1994, Sene et al. published MIR spectroscopy of cell walls, comparing primary cell walls from onion (*Allium cepa*), carrot (*Daucus carota*), rice (*Oryza sativa*), sweet corn (*Zea mays*), and polypogon (*Polypogon fugax steud*), many of which are important crops. Since then, the number of biospectroscopy studies of cell walls has increased significantly. Cell walls from various plant organs including leaves, roots, and fruits have been characterized (Largo-Gosens et al. 2014; Rösch et al. 2002; White et al. 2016). MIR spectroscopy has been used to study compositional changes resulting from growth and development, mutations in cell wall-regulating genes of cellulose/hemicellulose, and lignin, as well as the effects of biotic and abiotic stress (Kumar et al. 2016a; Largo-Gosens et al. 2014). These studies give insight into the molecular structure of plant cell walls and changes therein in response to stimuli and contribute to our fundamental understanding of this and closely related structures. Industrial-related processes such as spectral alterations associated with mechanical stress on plant cell walls, specifically cellulose and pectin orientation, were studied by Wilson et al. (2000). Recently, MIR and Raman spectroscopy were applied to characterizing non cellulose polysaccharides from cell wall fractions of tomato during development (Chylinska et al. 2016). Hence, plant cuticle and cell wall have been studied by biospectroscopy in both structure and function and under different conditions relevant to biological and industrial processes.

2.3.6 Cuticle and Cell Wall Together

Cuticle and cell walls are studied together when tissues such as leaves, and fruit are measured without manipulation (Figure 2.2). This is similar to studies on non-isolated cuticles, as part of whole plant organs such as freshly cut leaves. Analysis of leaves by MIR and Raman interrogates the cuticle, and palisade parenchyma of the adaxial leaf surface, respectively (Butler et al. 2015). Even ATR-FTIR spectroscopy, which penetrates only between 1 and 5 μm into the surface of plant structures, has been sufficient to effectively measure leaves from 32 tree species and identify them with good accuracy (Kazarian and Chan 2013; Ribeiro da Luz 2006). These studies demonstrate the versatility of biospectroscopy for cuticle and cell wall characterization separately and as combined layers as part of plant organs. Further work is needed to increase the study of plant cuticles and cell walls *in vivo*, and while biospectroscopy has contributed to this endeavor, analysis of whole plants, rather than freshly harvested leaves, remains elusive (Butler et al. 2015).

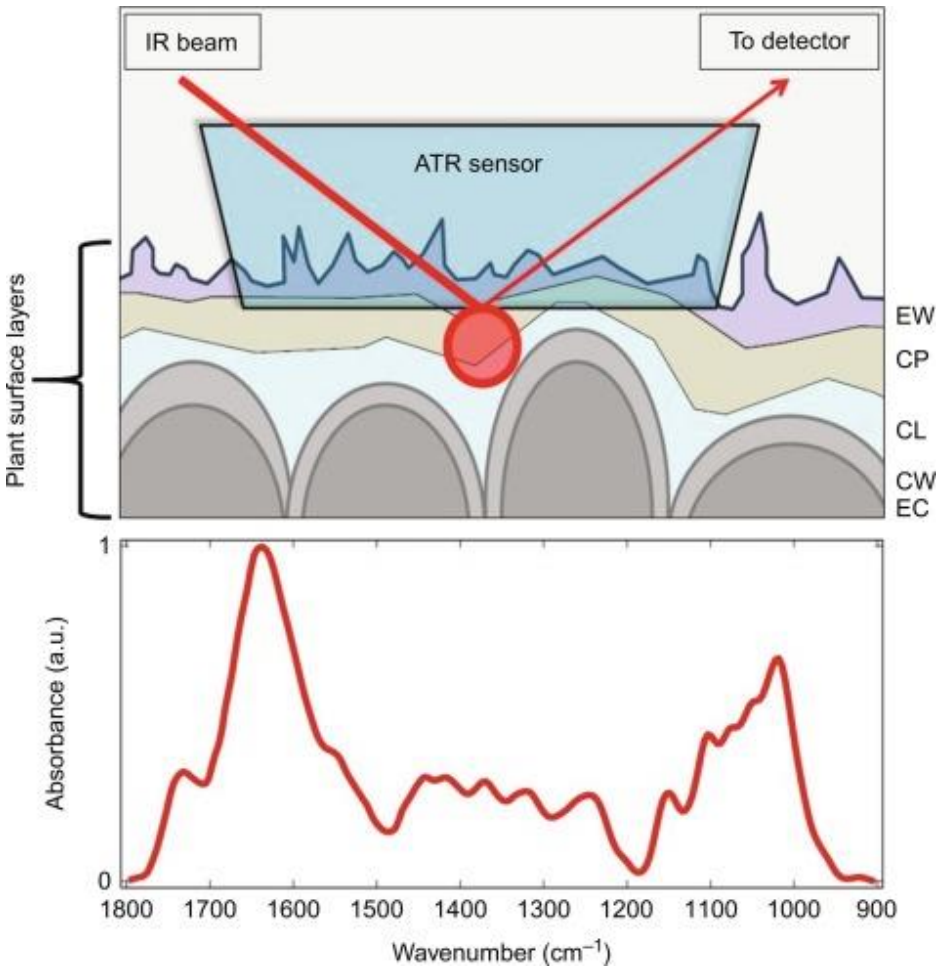

Figure 2.2 Top: Conceptual schematic of ATR-FTIR measurement on plant surface structures. CL, cuticluar layer; CP; cuticle proper; CW; cell wall; EC; epidermal cell; EW; epicuticular waxes. Bottom: Seventy averaged ATR-FTIR spectra of 4-week-old fully expanded tomato leaf (*Solanum lycopersicum* cv. Moneymaker) over the fingerprint region (1800–900cm^{-1}).

Work done thus far on the surface structure of plants will aid the development of biospectroscopy for examining the cuticle and cell wall within whole plants and fruit and how these layers change in response to various processes, both naturally and during crop cultivation. The ability to measure whole plant organs such as leaves and extract spectroscopic information relevant to physiological processes suggests that applications to crop monitoring in whole plants in the field or whole fruit before, at, or after harvest becoming a strong probability. Recent progress with methods such as ATR- FTIR and Raman spectroscopy has shown that surface structures

including cuticle and cell wall in leaves of whole plants and whole fruit can be measured without destructive effects entirely *in vivo* (Butler et al. 2015; Fu et al. 2016; Trebolazabala et al. 2013). It has been clear for more than two decades that biospectroscopy methods can contribute to our detailed understanding of important plant surface structures including the cuticle and cell walls in the lab, but development of biospectroscopy for complete *in vivo* analysis of plants will expand its application potential significantly into the area of commercial horticulture for physiological-based monitoring of plants and crops.

2.3.7 Plant-Environment Interactions

Growth and development of plants, in their natural habitat or in fields and greenhouses, is dictated in large part by their interaction with the external environment. When the environment becomes unfavorable, plants employ stress and defense responses to cope with suboptimal environmental conditions. In the light of climate change, the dynamic interaction between pathogens and the environment has become an important consideration (Nutter et al. 2010). Climate change and resulting effects likely influence the contribution of individual pests to annual crop loss (Figure 2.3).

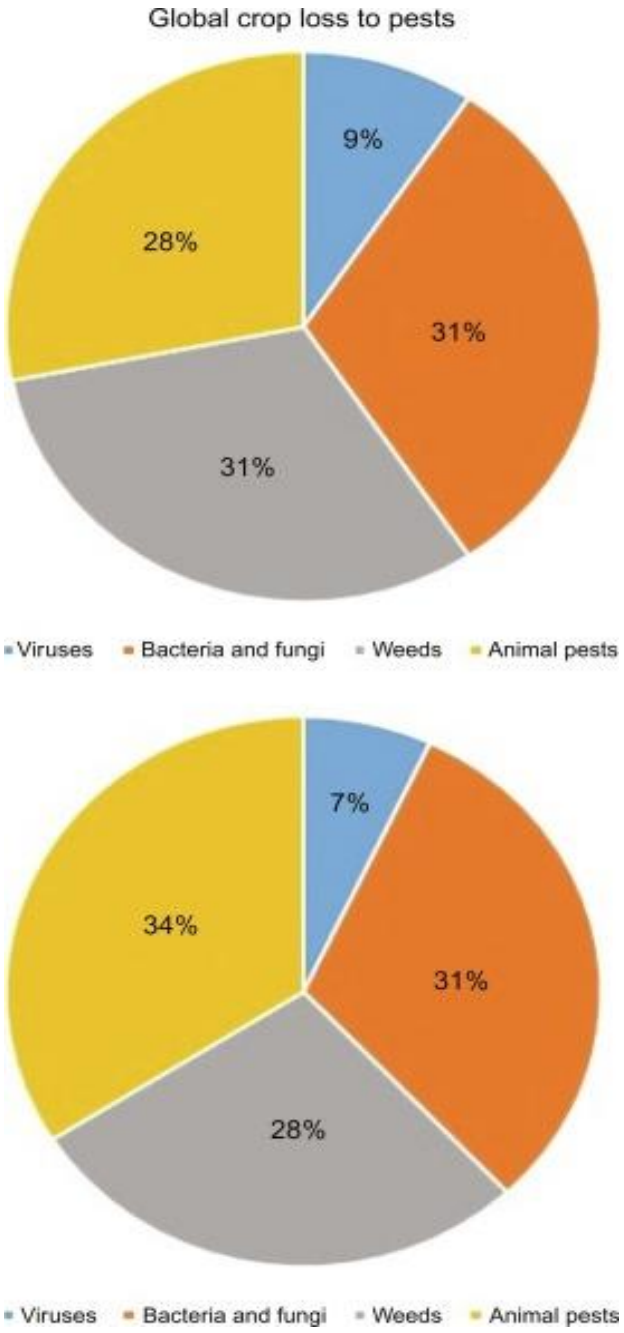

Figure 2.3 Contributions of pest and pathogen types to annual global crop loss. Top: crop loss to pests and pathogens for wheat, rice, maize, barley, potatoes, soybean, sugar beet, and cotton, for the years 1996–98. Bottom: crop loss to pests and pathogens for wheat, rice, maize, potatoes, soybean, and cotton for the years 2001–03 (adapted from Oerke and Dehne 2004, and Oerke 2006, respectively).

Both abiotic and biotic factors influence plant physiology, growth, and development and thus crop quality and yield (Suzuki et al. 2014). Pertinent abiotic factors influencing plants include drought, salinity, temperature, nutrient deficiency, phototoxicity, ozone (O_3), and anaerobic stresses (Suzuki et al. 2014) (see also Figure 2.1). Viruses, bacteria, fungi, nematodes, weeds, and herbivores are among the main biotic factors relevant to plant survival (Atkinson and Urwin 2012). Under natural or field conditions, combinations of abiotic and/or biotic stress occur where different stress types may positively or negatively affect each other (Atkinson and Urwin 2012). Furthermore, plant response to abiotic and biotic stressors overlaps on the molecular level, sharing general response elements such as signaling pathways, while differing in fine-tuned specific responses to individual stressors (Bostock et al. 2014; Kissoudis et al. 2014). Because stress responses precede plant disease, understanding the mechanisms of plant response to individual stresses and in combination could be especially useful for determining "biomarkers of stress effects," while plants are still in the reversible stage of stress (Bostock et al. 2014). During this acclimation stage, natural tolerance/resistance to stress prevent adverse effects, which once exhausted lead to irreversible strain, disease, and ultimately plant death. For precision crop protection and the prevention of plant disease caused by both abiotic and biotic stress, the preventative stages of plant disease, namely, the acclimation/resistance stage may be a specifically suitable target for biospectroscopy. Pre-symptomatic disease detection would have the added benefit of optimizing crop protection measures and reducing the overuse of harmful pesticides, which are becoming an increasing public concern. Detection and characterization of stress responses before irreversible damage ensues would be beneficial for several reasons including concomitant development of health monitoring tools. Elucidation of the intricacies of individual and overlapping stress responses by plant biologists would significantly aid in determining targets suitable as "stress biomarkers" in different crop species. Crop scientist in turn may be able to apply biological stress markers for early disease detection and identifying plants particularly resistant or tolerant to different stresses. Indeed, both disease detection and phenotyping are among the most important research areas for modern horticulture concerning plant and crop scientists collectively. Alternatively, direct detection of visually undetectable pests such as microscopic pathogens is also a priority, even though pathogen density is not necessarily indicative of disease severity for plants in the field (Mahlein 2016; Nutter et al. 2010). While direct pathogen detection may be difficult in

the field, it may be more readily applied to postharvest storage and transport of plant production, where infected or contaminated products pose potential health hazards. Thus, from the view of researchers, there is a desire to discern the details of individual and stress combinations, both abiotic and biotic, in order to identify key factors of plant stress early before damage is done. With the right technology, these key factors or biomarkers could help horticulturalists in their quest to reduce pre- and postharvest crop loss as a result of plant disease. It is noteworthy, that disease detection by default coincides with the development of health monitoring tools, as the control subjects used to study dis- ease are defined as healthy counterparts within individual studies.

Many applications of biospectroscopy have displayed its capacity to contribute to the areas of plant disease detection and health monitoring, through the investigation of biotic and abiotic stress, together with closely linked processes like leaf senescence. Linked to natural development, aging, and disease is the process of senescence, which has also been studied using biospectroscopy methods. The process of senescence is important because it is induced by several factors including stress and also occurs naturally as plants mature (Gepstein and Glick 2013). Leaf senescence has been studied in situ on detached leaves of black cherry (*Prunus serotina*), sweet pepper (*Clethra alnifolia*), *Capsicum annuum*, and *Nicotiana tabacum* using ATR-FTIR spectroscopy (Ivanova and Singh 2003). ATR-FTIR spectroscopy has also been used *in vivo* on leaves of whole plants to study plant growth and development including cell expansion and senescence (Butler et al. 2015).

Several main abiotic and abiotic stresses have been investigated in various plants including crops like wheat and tomato. Raman spectroscopy was applied to study mechanistic changes related drought stress induced by cutting leaves from spring wheat plants (Wesełucha-Birczynska et al. 2012); where drought stress in wheat is particularly relevant to horticultural challenges worldwide (Suzuki et al. 2014). Assessment of salt stress has been conducted on both plants and fruit. Salt-tolerant halophytes, ice plants (*Mesembryanthemum crystallinum*), where compared to *A. thaliana* under salt stress conditions and investigated using MIR spectroscopy (Yang and Yen 2002). Metabolic fingerprinting of tomato fruit extracts grown under high salt stress and normal conditions was investigated by Johnson et al. (2003). Salt stress and the effects on cell wall structure and leaf cell anatomy in coffee (*Coffea arabica*) have also been recently investigated (de Lima et al. 2014). Khairudin et al. (2014) investigated variable temperature on populations of the herb *Polygonum minus* and observed differences in important metabolites including flavonoids. Biospectroscopy to study nutrient stress has only been applied in specialized settings to our

knowledge. Interestingly, algae (*Micrasterias* sp.) have also served as a model system for biospectroscopy in which *in vivo* analysis, nutrient stress, and other anthropogenic abiotic stresses have been studied (Heraud et al. 2005; Patel et al. 2008). The effects of nutrient stress were studied using Raman spectroscopy on live algal cells *in vivo*, where the study was additionally used to compare data preprocessing effects on outcomes of computational analysis (Heraud et al. 2005). Transmission MIR using synchrotron radiation was employed on live and fixed tissue of the model plant Asiatic dayflower (*Commelina communis)* specifically to detect calcium deficiency (Butler et al. 2017). Metal stress was investigated through early effects of cadmium, and subsequent partial recovery, in clover (*Trifolium* sp.) leaves using MIR spectroscopy (Wei et al. 2009), as well as by Liu et al. (2014), who, similar to de Lima et al. (2014), correlated changes in cell wall structure with changes in leaf anatomy but in navel orange plants (*Citrus sinensis*). Ozone stress, as well as both biotic and abiotic stresses, was measured on sycamore tree (*Acer pseudoplatanus*) leaves exposed to air pollution, ozone, and fungal infection, giving potential mechanistic insight into shared stress responses such as reactive oxygen species generation (Ord et al. 2016).

Studies of biotic stresses, such as viruses, bacteria, and fungal pathogens, using biospectroscopy remain relatively limited to date, but progress has been made in the presymptomatic detection of disease. Investigations into biotic stress scenarios have also been selectively performed with several applications directed at pre-symptomatic disease detection and health monitoring in plants and trees. Biospectroscopy studies into biotic stress caused by fungal pathogens include the tar spot leaf fungus (*Rhytisma acerinum*) on Sycamore tree leaves by Ord et al. (2016). Plant–plant interference through metabolic profiling to study interspecies competition between a monocotyledon *Brachypodium distachyon* and a dicotyledon *A. thaliana* has been described by Gidman et al. (2003), with potential applications to weed pests.

Progress has been made in both plant health monitoring and disease detection, to demonstrate the applicability of biospectroscopy in these areas. Recently, MIR photoacoustic spectroscopy was used for pre-symptomatic detection of powdery mildew infection in *Rubus corchorifolius*, a Korean raspberry cultivar (Du and Zhou 2015). Previously, ATR-FTIR spectroscopy as a potential tool in huanglongbing and citrus variegated chlorosis diagnosis in leaves of sweet orange trees was explored (do Brasil Cardinali et al. 2012). Finally, and among the most recent developments has been the successful health monitoring of whole tomato plants. ATR-FTIR spectroscopy and Raman spectroscopy have been used, in combination, for the successful

and nondestructive monitoring of healthy plant growth and development in intact tomato plants (*Solanum lycopersicum*) (Butler et al. 2015). This study was performed entirely *in vivo* on plant leaves, where spectral alterations were consistent with major physiological processes including cell expansion in newer leaves and senescence in more mature leaves.

Taken together, the above studies show a significant contribution to an exceptionally diverse set of plant varieties and cultivars subject to various stress or disease conditions. Although as previously explained, the transfer of these studies to applied crop sciences requires further development of fully *in vivo* analysis. However, the progress to date prompts the further evaluation of biospectroscopy approaches to study plant–environment interactions, including effects of abiotic and biotic stresses, progression of disease, as well as complementary processes such as healthy development and natural senescence. Ultimately, the transition to a full nondestructive sensor technology will be met once certain technical limitations are overcome.

2.4 Challenges and Limitations

Development of biospectroscopy as an applied sensor technology requires portable instrumentation. There is no doubt that laboratory-based biospectroscopy instruments have contributed significantly to the plant laboratory, yet despite the availability of fully portable equipment, no commercial applications have been developed. Laboratory-based IR spectrometers rarely accommodate the analysis of whole plants, specifically in fields and semi controlled environments (CE rooms, glasshouses, etc.), and part of the reason why biospectroscopy applications require some form of sample preparation, limiting it as a potential field sensor. Fully portable instruments are available for biospectroscopy, and while these have existed for some time, they have to our knowledge gone without evaluation. For only a few examples of fully portable systems commercially available for material science and related purposes, see http://bwtek.com/technology/raman and https://www.agilent.com/en/products/ftir/ftir-compact-portable-systems for Raman and MIR spectroscopy, respectively. Both Raman and MIR spectroscopy offer variable attachments for customized uses. Thus, as part of the challenge of validating different instrument configurations, fully portable units currently advertised for more traditional material science applications must be evaluated for plant materials in the field. Especially because evaluating these instruments could significantly accelerate the development of fully portable biospectroscopy sensor systems for plants. Additionally, portable equipment

comparisons with laboratory-based spectrometers would contribute to the issue of validating tentative spectral biomarkers generated in the lab, under field conditions. Figure 2.4 shows challenges and considerations with regard to differences between laboratory and field, which are generally applicable to technologies hoping to bridge the gap between laboratory and field applications.

Figure 2.4 Differences in complexity between laboratory and field conditions showing considerations for knowledge exchange between laboratory-based and field-based systems. (adapted from Suzuki et al 2014).

The number of available options for sample preparation, spectral acquisition, and data analysis makes standardizing biospectroscopy approaches difficult leading to the need for extensive validation before technologies become commercially viable. This can be seen as analogous to the different conditions present within controlled laboratories compared very high variability seen under field conditions. Due to the versatility of biospectroscopy methods, most studies use different instruments and data analysis tools. Therefore, spectral wavenumbers, extracted and listed as part of a study, are influenced by the experimental design and instrument choice, which can make it difficult to compare different studies. Consequently, this may hinder the knowledge transfer and progression of biospectroscopy into routine application. In light of this, validation is needed to confirm spectral wavenumbers intended to act as "biomarkers of effect," many of which are tentatively assigned, due to the intrinsic ambiguity of extrapolating from individual bond vibrations to physiological effects. To augment the progression of biospectroscopy toward industry applications beyond its current uses, validation is required in combination with complementary methods ranging from traditional approaches like chromatography, to other optical

sensors and OMICs technologies (including genomics, proteomics, metabolomics, lipidomics, etc.) that rely on multicomponent analysis of various classes of biomolecules. In any case, before routine biospectroscopy applications are available, more standardized approaches, as well as combinatorial data analysis approach combining multiple lines of experimental inquiry, are imperative (Figure 2.5).

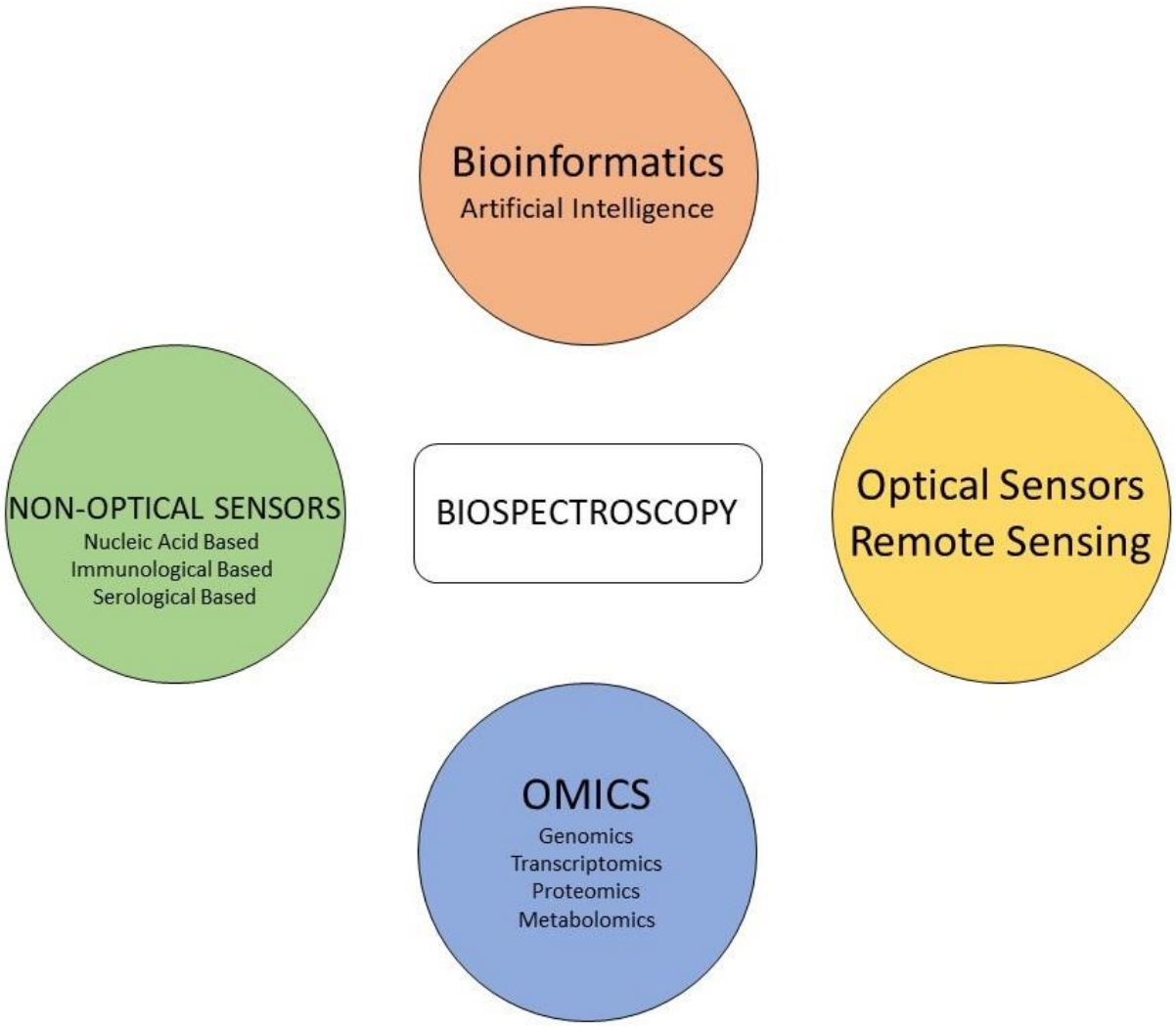

Figure 2.5 Complementary data for biospectroscopy validation and future integration of these technologies as part of a multi-sensor platform.

2.5 Conclusions and Future Perspectives

Biospectroscopy is suitably matched to study the fundamental processes important for crop protection and will help bridge the gap between laboratory and field applications by facilitating interdisciplinary research through knowledge transfer. To tackle the many challenges surrounding crop production and supply, innovative solutions to crop loss and food waste in the food system are paramount. Closely linked to crop protection is our understanding of the biological processes influencing plant development, as well as the details of plant composition as related to valuable plant substances. The natural complexity of plants, and the extraction of valuable substances for products, means that no single technology will fill the gap, to produce more with less, currently challenging modern agriculture. However, in meeting the challenge to fill the gaps leading to losses in plant resources, priority will likely be given to methods that yield highly specific data rapidly without destructive effects to delicate biological samples. To this end, many nondestructive optical sensor technologies are under evaluation, sharing common goals pertaining to crop protection and phenotyping (Mahlein 2016). As mentioned, these goals include disease detection; species identification for phenotyping and taxonomic classification; and increasing mechanistic insight into plant physiology and disease (Mahlein 2016). Further, Lucas (2011) (see also Crute 2003) outlined criteria for a truly sustainable technology in the context of agricultural productivity:

1. Based on the use of one or more renewable resources
2. Does not break down due to evolutionary change
3. Has a broad spectrum of applicability
4. Is affordable in the context of the local economy and crop value

It becomes immediately clear from this discussion and previous developments that biospectroscopy meets at least the first three points of these criteria. And while affordability is still to be determined in this context, biospectroscopy certainly remains a strong candidate sensor for development. The progression of biospectroscopy from its humble beginnings in the analytical and material science laboratories, for basic compound analysis, has expanded rapidly into the areas of plant and crop sciences for the analysis of complex biological materials. The many functions of biospectroscopy in the plant laboratory, whose principles potentially extend to applications in the field, are plentiful. Biospectroscopy has demonstrated its capacity to meet many of the criteria

needed to fulfill specific goals within the plant, crop, and food sciences, which has warranted its development toward an applied sensor technology for industry. Despite scarce applications to intact plant systems *in vivo*, there remains exciting potential for biospectroscopy especially in expanding these applications. Once challenges, including validation of fully portable instruments, are successfully overcome, through integration of biospectroscopy data with complementary methods, it may become a broadly applicable and commercially available technology for analysis and diagnosis.

While most biospectroscopy studies analyze primarily solid or semisolid samples, there are future prospects in other prominent areas of plant and crop science, which would benefit from gas and liquid sampling modes of IR spectroscopy. Specific examples here include the analysis of VOCs such as isoprene, which have become compounds of interest, due to their apparent ecological and defense functions (Dudareva et al. 2013). Liquid-based biospectroscopy may prove useful for the investigation of changes in the composition of plant biofluids, xylem and phloem sap. Biofluid analysis is currently a major topic in the biomedical area of biospectroscopy (Baker et al. 2016b), where "liquid biopsies" performed on plants may be a knowledge exchange opportunity between plant and biomedical sciences. Another area of interest will be the development and combination of artificial intelligence with biospectroscopy, where computers will autonomously take on all data analysis and processing without any external subjective influence (Figure 2.5).

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Chapter 3: Determination of Developmental and Ripening Stages of Whole Tomato Fruit using Portable Infrared Spectroscopy and Chemometrics

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Abstract

Background: Development and ripening of tomato (*Solanum lycopersicum*) fruit are important processes for the study of crop biology related to industrial horticulture. Versatile uses of tomato fruit lead to its harvest at various points of development from early maturity through to red ripe, traditionally indicated by parameters such as size, weight, color, and internal composition, according to defined visual 'grading' schemes. Visual grading schemes however are subjective and thus objective classification of tomato fruit development and ripening are needed for 'high-tech' horticulture. To characterize the development and ripening processes in whole tomato fruit (cv. Moneymaker), a biospectroscopy approach is employed using compact portable ATR-FTIR spectroscopy coupled with chemometrics.

Results: The developmental and ripening processes showed unique spectral profiles, which were acquired from the cuticle-cell wall complex of tomato fruit epidermis *in vivo*. Various components of the cuticle including cutin, waxes, and phenolic compounds, among others were identified, as well as from the underlying cell wall such as celluloses, pectin and lignin like compounds. Epidermal surface structures including cuticle and cell wall were significantly altered during the developmental process from immature green to mature green, as well as during the ripening process. Changes in the spectral fingerprint region (1800-900 cm^{-1}) were sufficient to identify nine developmental and six ripening stages with high accuracy using support vector machine (SVM) chemometrics.

Conclusions: The non-destructive spectroscopic approach may therefore be especially useful for investigating *in vivo* biochemical changes occurring in fruit epidermis related to grades of tomato during development and ripening, for autonomous food production/supply chain applications.

Key Words: Tomato, Development, Ripening, Crop biology, MIR spectroscopy, Chemometrics

3.1 Background

Global food security relies on the combination of effective crop production, distribution, and utilization (Berners-Lee et al. 2018). Crop production and distribution are both becoming increasingly challenging whilst population growth and changes in climate are leading to food shortages and malnutrition worldwide (IFPRI 2017). Conventional farming practices have struggled to increase the production of major crops worldwide (Ray et al. 2013). Due to lack of available land for food production, it is expected that much of the increase in crop production will occur through higher yields, intensified cropping and a reduction of waste in the supply chain due crop loss to climate, pests, pathogens, as well as downstream consumer waste (FAO 2009; Godfray et al. 2010). Innovative solutions that maximize crop production and reduce waste are therefore of paramount importance to maintaining food security. While numerous approaches to aid with this are being developed, technology-based solutions to farming are frequently confounded by the large number of crop species (and cultivars) grown and the complexity of plant-environment interactions within crop production systems. Therefore, there is an urgent need for the development of novel approaches for improving our understanding of crop biology and the development of applied farming tools to maximize production, minimize losses and to improve pre- and post-harvest production and utilization.

Tomato (*Solanum lycopersicum*) is one of the most important crops globally valued at 124.6 billion US dollars annually (FAO 2017) representing the largest sector of the fleshy fruit market (Bapat et al. 2010; FAO 2017). It is widely used as plant model due to its short generation time, and well-studied genetic, biochemical, and physiological properties (Tomas et al. 2017). Rich in beneficial phytochemicals, tomato fruit are delicate, develop and ripen quickly and are used at various stages of their development either whole, or for various processing purposes including canned goods, pastes, sauces, juices, etc. (Thakur et al 1996). Each of these products require fruit at different stages of development or ripening ranging from early immature to red ripe fruit depending on the number of days post anthesis (dpa) (Thakur et al 1996). The development and ripening of tomato fruit both of which are parameters important to the horticultural industries influencing fruit quality and shelf life (Bapat et al. 2010; Lara et al. 2014). The ability to accurately and non-destructively monitor changes occurring during tomato fruit development and ripening are therefore of utmost interest to both plant biologists and horticulturalists.

The plant epidermal layers and associated surface structures provide the plant-environment interface necessary for maintaining plant integrity, regulating fruit growth, and determining shelf life (Lara et al. 2014; Segado et al. 2016). Tomato fruit epidermis is composed of an integrated heterogeneous multi-layered matrix including the cuticle (cuticle proper and cuticular layer), cell wall, and epidermal cells (Yeats and Rose 2013). These layers undergo extensive changes during fruit development and ripening. However, to date the molecular mechanisms involved and how these changes influence characteristics like morphology, texture, pathogen susceptibility and shelf life have not been elucidated fully (Yeats and Rose 2013). In addition, it has been difficult to study the cuticle and cell wall separately due to the recalcitrant nature of these tissues (Dominguez et al. 2015). Therefore, novel approaches to investigate plant surface structures are essential to determine how they contribute to the healthy growth and development, or appearance of abnormal conditions, in horticultural products. Furthermore, these approaches need to be translatable into practical field-based applications to have relevance to both fundamental plant biology studies and applied crop science. Although there are a number of analytical tools, traditionally used in the laboratory which might be suitable for field-based horticultural applications (Mahlein 2016; Martinelli et al. 2015; Sankaran et al. 2010), the tools available to study plant surface structures non-destructively are limited.

Optical sensors based on light-matter interactions have been implicated as effective tools for the non-destructive monitoring of plant health and disease detection based on spectral signatures (Butler et al. 2015; Mahlein 2016). Particularly mid infrared (MIR) vibrational spectroscopy combined with chemometrics has been widely used as a bioanalytical tool that offers non-destructive analysis of most types of samples (Skolik et al 2018b). Vibrational spectroscopy, also known as surface techniques, typically probes the surface layers of samples to micrometer depths and, due to advancements in data analysis, can also be used to analyze complex heterogeneous biological samples, termed biospectroscopy. The unique spectrum of biological materials between 4000-400 cm^{-1} (2.5-25 μm wavelengths), produced through light-matter interactions between the IR radiation and the sample, contains biochemically specific variables useful for biological applications (Kazarian and Chan 2013). Many biological materials absorb preferentially in the 'fingerprint region' (1800-900 cm^{-1}), therefore this region is often the spectral range selected for analysis (Martin et al. 2010). The analysis of spectral data can be divided into exploratory and diagnostic analyses (Trevisan et al. 2012). Exploratory data analysis includes data

visualization, pattern recognition, and biomarker extraction (Kelly et al. 2011; Trevisan et al. 2012). Examples of analysis models used for these purposes include unsupervised learning such as principal component analysis (PCA), and supervised methods such as linear discriminant analysis (LDA) (Trevisan et al. 2012). Diagnostic data analysis aims at evaluating classifier performance for autonomous decision making. Various classifiers commonly used include LDA, support vector machine (SVM), naïve Bayes, and artificial neural networks (ANN), each of which exhibits varying levels of model complexity. MIR spectroscopy together with specialized data analysis have been applied to address important horticultural issues including plant health monitoring, plant-environment interactions, disease detection, phenotyping, and taxonomic relationships (Butler et al. 2015; Ord et al. 2016; Zimmermann et al. 2015). However, the development of biospectroscopy-based bioanalytical approaches for crop science that allow plants to be studied both in the lab and in a field-environment is essential for its wider adoption as a horticultural tool (Skolik et al. 2018b).

Currently, portable Raman spectrometers, which can measure intact samples are more readily available than IR spectrometers with such a capability. Consequently, to date, progress towards the development of biospectroscopy-based bioanalytical approaches for the analysis of intact crops has been limited primarily to the use of Raman spectroscopy, although this technique has only been recently employed for whole sample analysis (Farber and Kurouski 2018; Fu et al. 2016; Trebolazabala et al. 2013, 2017). Several other techniques outside the MIR range such as near-IR (NIR), ultraviolet (UV) and visible light, as well as hyperspectral analysis have been used to assess quality parameters in tomato (Huang et al. 2017; Lu et al. 2017; Sirisomboon et al. 2012). However, few of these studies provide detailed biochemical insight into the changes occurring *in vivo* during development and ripening and have traditionally focused solely on classification performance or correlation between traditional quality parameters and spectral data (Bureau et al. 2016). Furthermore, the potentially small measurement area, as well as the higher energy of NIR, UV, visible, and Raman instruments, increases the light penetration depth into the sample over a very small area making it potentially difficult to obtain reliable biological information. MIR spectroscopy in contrast offers sampling modes with very well-defined measurement areas and light penetration depths (Kazarian and Chan 2013), which permit biochemical investigations when combined with known chemical compositions of plant tissues under investigation (Heredia-Guerrero et al. 2014; Skolik et al. 2018a). Attenuated total reflectance Fourier transform (ATR-

FTIR) spectroscopy is one method with a very well-defined light penetration depth, where macro measurements over larger areas are possible (Kazarian and Chan 2013). In other fields, ATR-FTIR has proved exceptional at providing both biochemical insight into biological samples, as well as providing strong discriminating power in combination with classification models (Martin et al. 2010; Skolik et al. 2018a). This suggests a need to evaluate the use of Raman complementary methods such as reflectance spectroscopy including ATR-FTIR within crop science. In order to increase the capacity for spectroscopy-based methods to provide biochemical information as well as classification performance, it is imperative to assess complementary approaches aimed at developing multi-sensor platforms, which will be required for complex systems.

Tomato is widely used as a model system for studying cuticle, cell wall, and epidermis during fruit development and ripening. In the present study therefore, we apply a novel approach combining multivariate chemometrics for biomarker extraction and assessment of classification performance. Biomarker extraction as part of a two-tiered approach is aimed at studying the effects of development and ripening on the spectral signatures of tomato fruit. First, exploratory multivariate analysis in the form of PCA-LDA was used to extract tentative wavenumber biomarkers associated with differences in the nine developmental stages of tomato fruit from 4-36 dpa, and subsequently the six distinct ripening stages from mature green to red ripe tomato (approx. 34-55 dpa). Biochemical entities identified as biomarkers are explored. The second tier involves SVM classification of the nine developmental and six ripening stages, in order to determine the potential for autonomous grading of tomato fruit maturity and ripening stages based on MIR fingerprint spectra.

3.2 Results

3.2.1 Spectral Analysis of Tomato Fruit Development

Tomato fruit development and ripening were split into two distinct processes, as shown visually in Figure 3.1. Spectra were acquired from each developmental timepoint including ripening. Figure 3.2 shows the class mean raw and pre-processed fingerprint spectra for the development (Figure 3.2A and B) and ripening (Figure 3.2C and D) processes. Figure 3.2 clearly shows that most sharp absorbance peaks are evident within the fingerprint region between 1800

and 900 cm^{-1}. This region holds most of the biochemical information pertaining to the samples and was therefore the focus of the investigation.

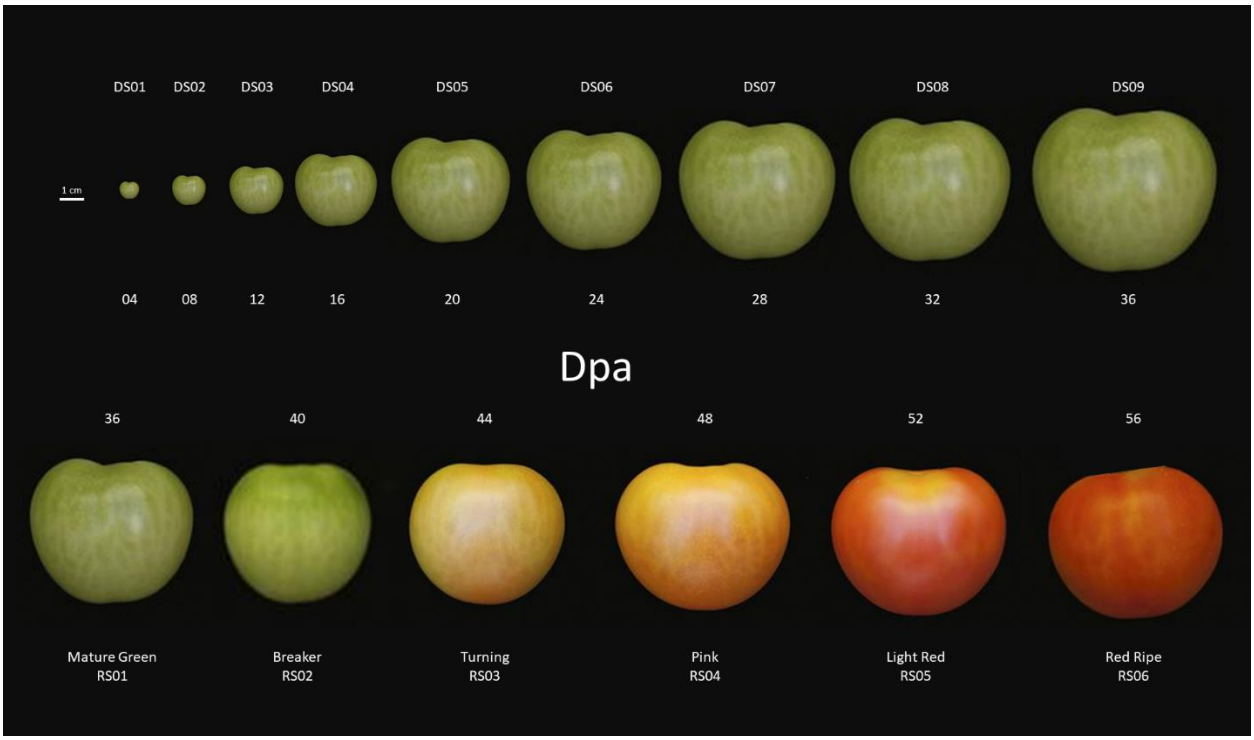

Figure 3.1 Tomato fruit of *Solanum lycopersicum* cv. Moneymaker: developmental (top) and ripening (bottom) stages used as individual groups for MIR ATR-FTIR spectral analysis; dpa (days post anthesis).

Linear discriminant analysis effectively distinguishes tomato fruit development based on PCA factors. Figure 3.3 shows the three linear discriminants LD1, LD2, and LD3 respectively, based on LDA of PCA factors. Variable separation was observed along the three LDs, of spectral clusters belonging to the nine different times of development. Discriminant function 1 (LD1) was effective at separating developmental stages, although clear separation of DS02 from DS03, DS05 from DS06, and DS07 from DS08 was not observed (Figure 3.3A). This indicates that spectral features of these stages show little to no differences with respect to the other developmental classes (DS01, DS04, and DS09). While DS02/DS03, DS05/DS06, and DS07/DS08 formed distinct clusters with no clear separation, these pairs were very distinct from one another effectively forming six distinguishable groups along LD1 (Figure 3.3A). In contrast, discriminant LD2

showed a definitive separation of DS02 and DS03 but not of adjacent DS05/DS06 or DS07/DS08 (Figure 3.3B). Separation of DS05 from DS06 was achieved along LD3 as opposed to no observable separation between DS07 and DS08 (Figure 3.3C). Based on spectral data, it appears that DS07 and DS08 were most closely related as indicated by multivariate PCA-LDA of the first three LDs shown in entirety in Figure 3.3. This is likely due to minimal changes occurring in the last few days of tomato fruit maturation, compared to changes occurring well before the mature green stage.

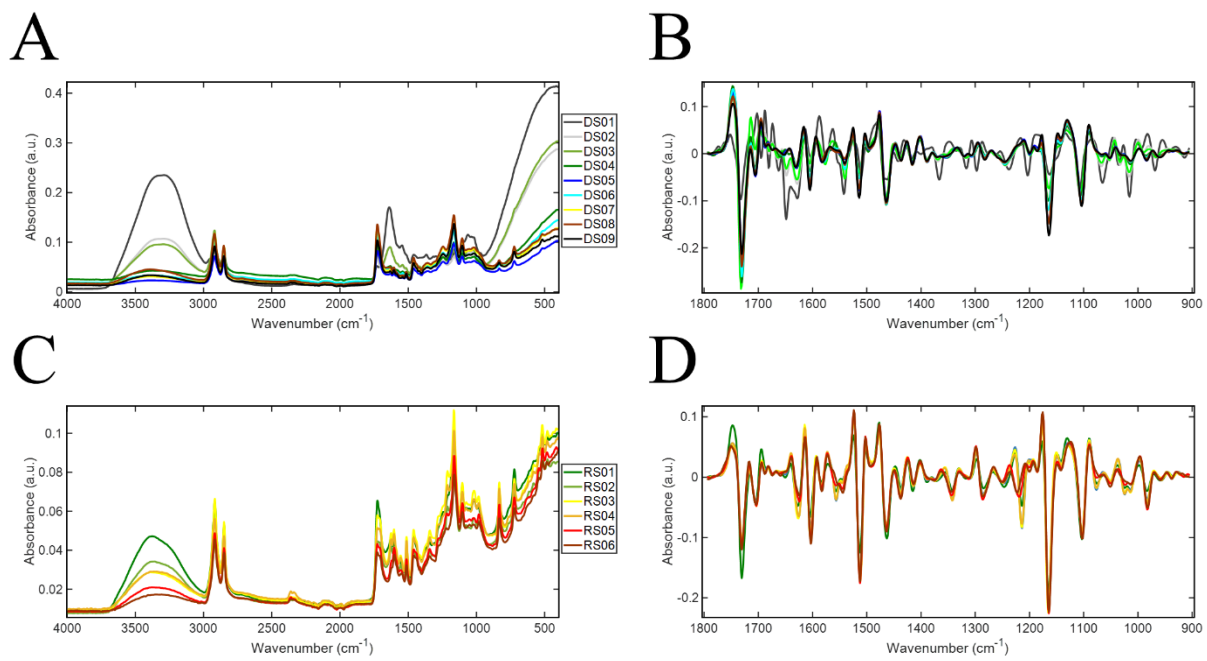

Figure 3.2 ATR-FTIR spectra as class means with raw and pre-processed spectra for development (A and B) and ripening (C and D).

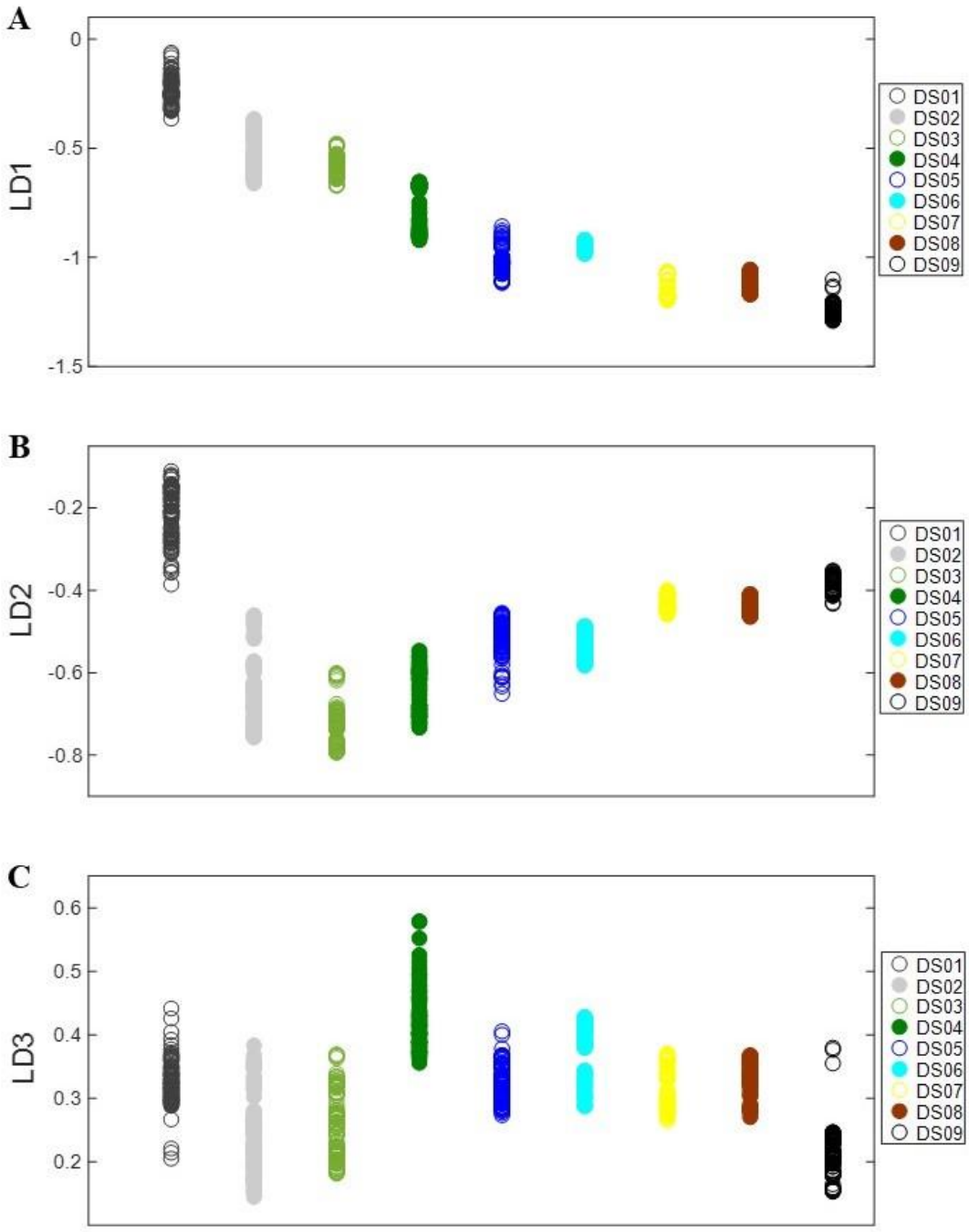

Figure 3.3 PCA-LDA 1-dimensional scores plots of tomato fruit developmental stages (DS01-DS09) along LD1 (A), LD2 (B), and LD3 (C).

In order to explore further the group clustering observed in the 3-dimensional discriminant space, PCA-LDA loadings were extracted for each of the three LDs to determine the specific spectral alterations associated with the tomato fruit developmental process. This provides a summary of the main biochemical changes occurring during tomato fruit development from DS01-DS09 between 4 and 36 dpa. Figure 3.4 shows PCA-LDA loadings (LD1-LD3) representing the main qualitative wavenumbers discriminating developmental stages of tomato fruit. The top six wavenumber biomarkers were selected from each loading to qualitatively characterize the biochemical compounds showing the greatest changes. Biomarkers extracted via PCA-LDA loadings provide potential biochemical and molecular markers for monitoring fruit development. Table 3.1 shows the top six discriminating wavenumbers for each of LD1-3 representing the main biochemical functional groups and associated compounds accompanying the developmental process in this cultivar. Specific changes were observed in the wavenumber regions 1732-1714, 1698-1627, 1558-1511, 1467-1464, 1173-1102, and 1017 cm^{-1}.

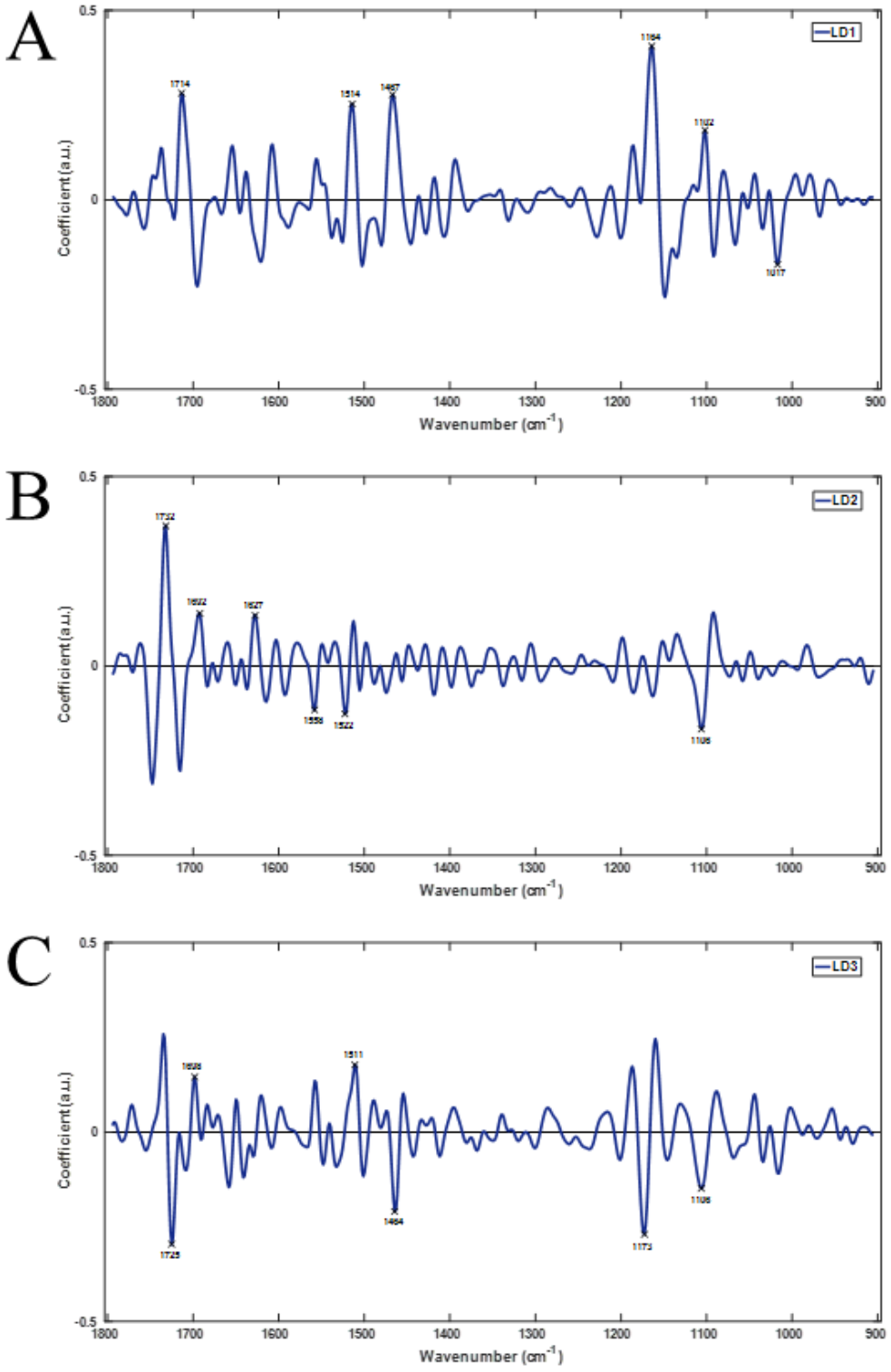

Figure 3.4 PCA-LDA loadings from the first three LDs; LD1 (A), LD2 (B), and LD3 (C) showing the top six discriminating wavenumbers responsible for group clustering of LD scores from developing tomato fruit (DS01-DS09).

Table 3.1 Top six discriminating wavenumbers, corresponding vibrational modes, and biochemical assignments for the first three LDs as indicated by individual loadings of tomato development.

PCA-LDA Loadings	Wavenumber (cm^{-1})	Vibrational Mode	Biochemical Assignment
LD1	1714	ν(C=O· · ·H) ester ν(C=O)	Cutin Phenolic compounds, pectin
	1514	ν(C-C) aromatic Amide II, ν(C=N), ν(C=C)	Phenolic compounds Proteins Lignin
	1467	δ(CH_2) scissoring	Cutin, glycerolipids, wax hydrocarbons
	1164	ν_a(C-O-C) ester ν(C-OH), ν(C-O-C)	Cutin Polysaccharide, cellulose
	1102	ν_s(C-O-C) ester ν(C-O) $\nu_a(PO_2)$	Cutin Pectin, cellulose, carbohydrates Phosphate
	1017	ν(C-O), ν(C-C) ν(C-OH)	Pectin, cellulose Pectin
LD2	1732	ν(C=O) ester ν(C=O)	Cutin, Lignin, wax, suberin-like aliphatic compounds
	1692	ν(C=O· · ·H weak) ν(C=O· · ·H strong)	Cutin Cutin
	1627	ν(C=C) phenolic acid ν(C=O) Amide I	Phenolic compounds De-esterified pectin Proteins

	1558	ν(C=C) phenolic acid	Phenolic compounds Proteins
	1522	ν(C-C) aromatic Amide II, ν(C=N), ν(C=C)	Phenolic compounds Proteins Lignin
	1106	ν_s(C-O-C) ester ν(C-O) $\nu_a(PO_2)$	Cutin Cellulose, pectin, carbohydrates Phosphate
LD3	1725	ν(C=O) ester	Cutin, pectin, phenolic compounds
	1698	ν(C=O· · ·H strong) ν(C=O· · ·H weak)	Cutin Cutin
	1511	ν(C-C) aromatic	Phenolic compounds Lignin
	1464	$\delta(CH_2)$ scissoring	Cutin, glycerolipids, wax hydrocarbons
	1173	ν_a(C-O-C) ester	Cutin
	1106	ν_s(C-O-C) ester ν(C-O)	Cutin Pectin, cellulose, carbohydrates

(Wavenumber references: Butler et al. 2015, 2017; Heredia-Guerrero et al. 2014; Largo Gosens et al. 2014; Movasaghi et al. 2008; Ord et al. 2016).

3.2.2 Spectral Analysis of Tomato Fruit Ripening

Similar to multivariate analysis of developmental stage, ripening stages of tomato were also effectively distinguished along LD1, LD2, and LD3 (Figure 3.5). These LDs were variably effective at separating the six distinct ripening stages. Most significant class separation was observed along LD1, with the six ripening stages showing clear separation, except RS03 and RS04, which showed no separation and were thus the most similar among these groups (Figure 3.5A).

Although LD1 was unable to separate RS03 from RS04, LD2 was highly effective at distinguishing RS03 from RS04 and all other ripening stages (Figure 3.5B). Variable group clustering was seen along LD3, where RS05 was most clearly separated from other groups (Figure 3.5C). As with tomato fruit development, ripening stages showed unique class clustering along the different LDs, indicating spectral features unique to each class. This raises the intriguing possibility that different LDs may be used in a targeted way to identify ripening stages in addition to different developmental stages based on this methodology.

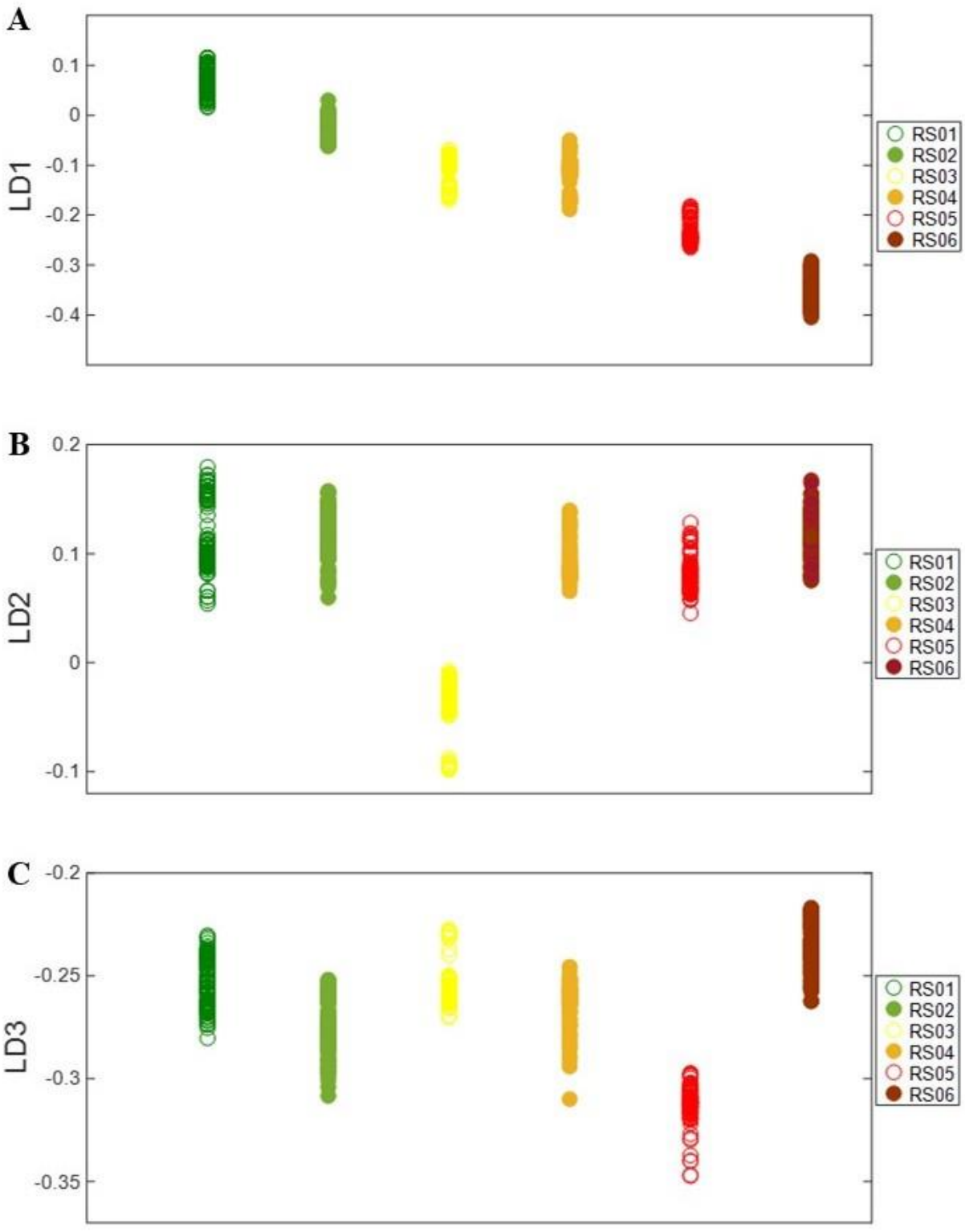

Figure 3.5 PCA-LDA 1-dimensional scores plots of tomato fruit ripening stages (RS01-RS06) along LD1 (A), LD2 (B), and LD3 (C).

Spectral biomarkers for ripening extracted through PCA-LDA loadings, identified similar cuticle and cell wall components to those identified during development (Figure 3.6); cutin, phenolic compounds, lipids, and waxes were identified at wavenumbers 1721, 1719, 1632, 1603 1561, 1473, 1160, and 1156 cm^{-1} whilst lignin-like compounds, cellulose, pectin, and other polysaccharides were identified at wavenumbers 1719, 1603, 1539, 1504, 1239, 1160, 1156, 1078, and 1041 cm^{-1} (Table 3.2). In addition, several unique spectral biomarkers related to proteins and indicative of ripening were identified, including the prominent Amide I, II, and III regions. Proteins are prominent components of the cell wall, and to lesser extent plant cuticles, suggesting that these proteins may be ripening-dependent based on the multiple protein vibrations seen over the fingerprint spectrum. Protein vibrational modes were seen specifically at wavenumbers 1632, 1575, 1539, 1239, and 1218 cm^{-1} (Table 3.2). These changes may be associated with the softening of the fruit skin and the depolymerization of pectin and other natural polymers during ripening (Brummell and Harpster 2001) resulting in alterations to both the accessibility and abundance of proteins embedded in the cell wall-cuticle complex. The (C-H) vibration at 1504 cm^{-1} is potentially linked to an increase in the carotenoid content, specifically lycopene, during ripening. The ripening-specific biomarker at 1078 cm^{-1} has previously associated with xyloglucan and is also likely to be associated with xyloglucosyltransferase/endohydrolase (XTH) activity in the epidermis, which has an active role in fruit softening in tomato (Miedes et al. 2010). Interestingly, wavenumber 1041 cm^{-1} was associated with arabinogalactan. Arabinogalactan-glycoproteins at the plant cell surface have been implicated in plant growth and development and may integrate changes occurring in the cell wall and cuticle layers during ripening (Ellis et al. 2010; Lamport and Varnai 2013). The detection of protein vibrations, which may signify increased protein abundance during tomato ripening, reinforces the identity of arabinogalactan as part of glycoproteins, and provides a link between xyloglucan and XTH enzyme activity during the ripening program.

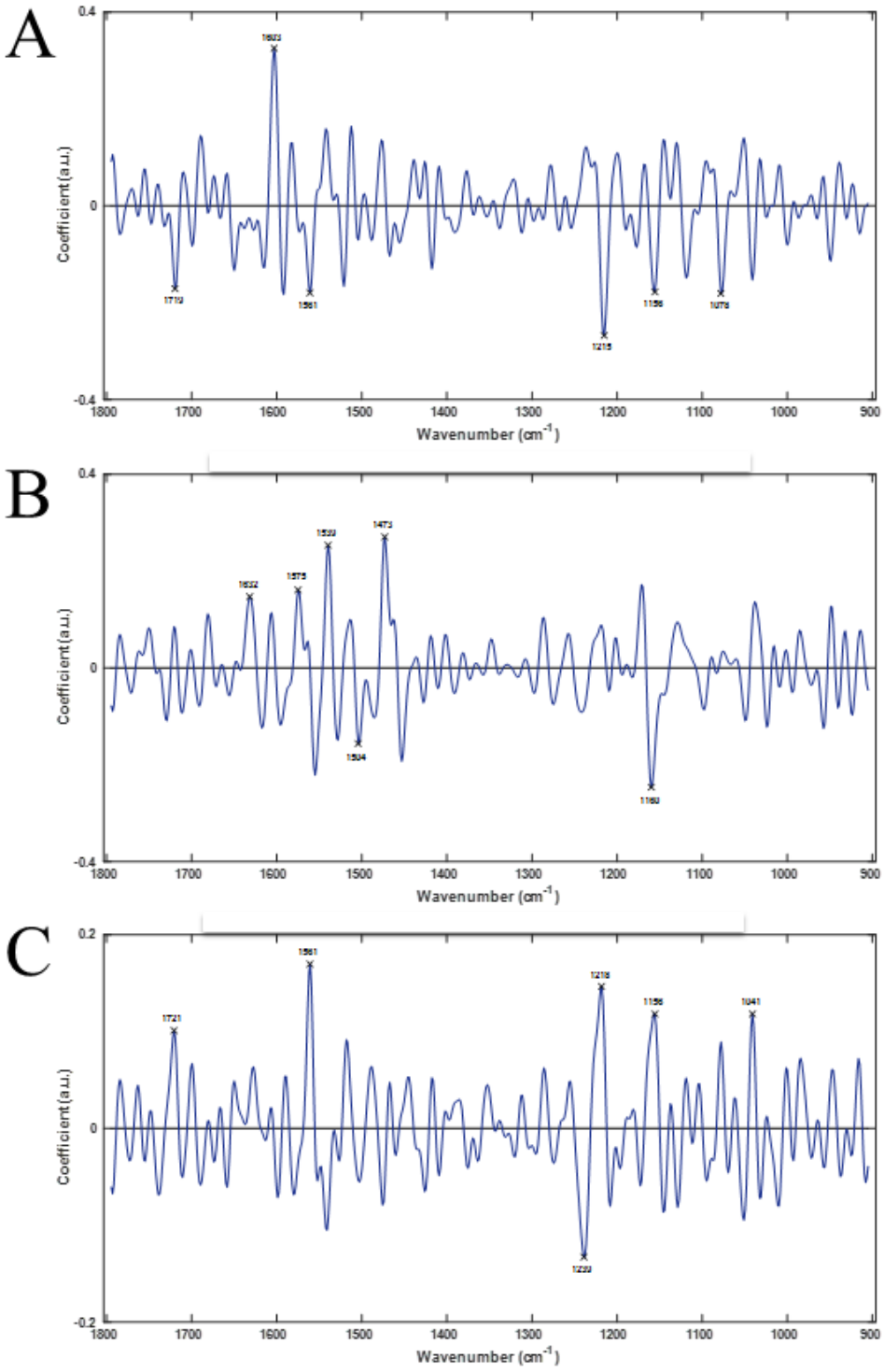

Figure 3.6 PCA-LDA loadings from the first three LDs; LD1 (A), LD2 (B), and LD3 (C) showing the top six discriminating wavenumbers responsible for group clustering of LD scores from ripening tomato fruit (RS01-RS06).

Table 3.2 Top six discriminating wavenumbers, corresponding vibrational modes, and biochemical assignments for the first three LDs as indicated by individual loadings of tomato fruit ripening.

PCA-LDA Loadings	Wavenumber (cm^{-1})	Vibrational Mode	Biochemical Assignment
LD1	1719	ν(C=O· · ·H) ester ν(C=O)	Cutin Pectin, lipid, polysaccharides, phenolic compounds
	1603	ν(C-C) aromatic ν(COO), ν(C=C)	Phenolic compounds Pectin, lignin
	1561	ν(C-C) aromatic	Phenolic compounds
	1215	$\nu_a PO_2$ Amide III	Phosphate Proteins
	1156	ν(C-OH) ν_a(C-O-C) ester	Cellulose, polysaccharide Cutin, pectin
	1078	ν(C-O), ν(C-C) ν(C-OH) $\nu_s PO_2$	Xyloglucan Oligosaccharide Phosphate
LD2	1632	ν(C=C) phenolic acid ν_s(C-C) ring Amide I	Phenolic compounds Cellulose Proteins
	1575	ν(C=N) ν(C-C) phenyl group	Proteins Phenolic compounds
	1539	Amide II ν(C=N)	Proteins Lignin
	1504	ν(C=C) ν(C-H) Amide II	Lignin Carotenoid Protein

	1473	$\delta(CH_2)$ scissoring	Glycerolipids, wax hydrocarbons
	1160	ν_a(C-O-C) ester ν(C-OH), ν(C-O-C)	Cutin Cellulose, polysaccharide
LD3	1721	ν(C=O) ester ν(C=O)	Cutin, pectin Phenolic compounds, lipids, polysaccharides
	1561	ν(C-C) aromatic	Phenolic compounds
	1239	Amide III ν(C-O) $\nu_a PO_2$	Proteins Pectin Cellulose / hemicellulose Phosphate
	1218	Amide III $\nu_a PO_2$	Proteins Phosphate
	1156	ν_a(C-O-C), ester ν(C-OH), ν(C-O-C)	Cutin Polysaccharide, cellulose
	1041	ν(C-O), ν(C-C) ν(O-CH_2)	Cellulose Arabinogalactan

(Wavenumber references: Butler et al. 2015, 2017; Heredia-Guerrero et al. 2014; Largo Gosens et al. 2014; Movasaghi et al. 2008; Ord et al. 2016).

3.2.3 Autonomous Determination of Tomato Fruit Development and Ripening Stages

Classification using PCA-LDA was satisfactory for the tentative assignment of spectral biomarkers but SVM was required for more effective classification of developmental and ripening stages. Autonomous sorting of tomato fruit based on their spectral characteristics is an exciting possibility. To test the feasibility of using classification performance based on fingerprint spectra, both PCA-LDA and SVM were applied (Table S3.1-S3.3; Figure S3.1-S3.2). While the classification performance of PCA-LDA was satisfactory (Table S3.1), this approach was used primarily for biomarker extraction. To improve classification performance from that achieved by PCA-LDA, SVM was applied. While SVM was superior for sheer classification purposes, SVM

is a non-linear method and therefore does not provide biochemical information. Table 3.3 shows the results for SVM based autonomous classification of developmental stages based on ATR-FTIR fingerprint spectra. High accuracy was observed for all developmental grades of tomato, with minimal misclassification only between directly related late stages of development (DS08 and DS09) between ~34-36 dpa as shown in the confusion matrix for development (Table 3.3). Sensitivity and specificity rates for development were correspondingly high (Table S3.2 and Figure S3.1). These results indicate that changes in the epidermal surfaces are sufficient to determine with exceptionally high sensitivity and specificity, the developmental stage of whole tomato fruit non-destructively with compact equipment. Further, tomato development can be distinguished, in this case within ± 4 days of the next developmental stage.

Table 3.3 Confusion matrix showing predictive performance calculated for the SVM chemo-metric model intended to differentiate tomato fruit developmental stages from their ATR-FTIR spectral data.

	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%

Tomato fruit are harvested at different developmental stages depending on their end use to ensure the desired qualities unique to each developmental. There are at least 4 maturity grades of tomato harvested between 4 to 36 dpa (M1-M4) providing fruit of different quality at maturity (Maul et al. 1998; Sargent 1997). Currently, the horticultural industry typically relies on subjective visual and/or destructive determination of maturity and ripening stages for tomato grading (Kader

and Morris 1976). Therefore, the development of objective and non-destructively approaches to determine fruit maturity and ripening stage, while gaining valuable biochemical information, would be beneficial to the industry. Here we provide evidence that ATR-FTIR combined with chemometric modelling can classify many distinct developmental stages, in this case nine, without destructive measurement but high selectivity and specificity (Table 3.3 and Table S3.2). These results exceed or are at least on par with other spectroscopic approaches currently used to assess fruit maturity and quality parameters (Bureau et al. 2016; Huang et al. 2018; Lu et al. 2017).

Six horticultural ripening grades are typically distinguished based on color schemes; mature green, breaker, turning, pink, light red, and red (Sargent 1997). Spectral data combined with chemometrics was also effective at identifying the six distinct ripening grades of tomato. As with developmental groups, spectra of the six ripening grades were subjected to SVM analysis (Table S3.3 and Figure S3.2). Table 3.4 shows that the six ripening grades were distinguished with almost between 99 and 100% accuracy, the only exception being between the adjacent ripening grades ‘turning’ and ‘pink’.

Table 3.4 Confusion matrix showing predictive performance calculated for the SVM chemometric model intended to differentiate tomato fruit ripening stages from their ATR-FTIR spectral data.

	Mat. Green	**Breaker**	**Turning**	**Pink**	**Light Red**	**Red Ripe**
Mat. Green	100%	0%	0%	0%	0%	0%
Breaker	0%	100%	0%	0%	0%	0%
Turning	0%	0%	99%	1%	0%	0%
Pink	0%	0%	0%	100%	0%	0%
Light Red	0%	0%	0%	0%	100%	0%
Red Ripe	0%	0%	0%	0%	0%	100%

3.3 Discussion

3.3.1 Spectral Characteristics of Tomato Fruit Development

Discriminant analysis reveals class-dependent clustering of spectral groups and allows the extraction of qualitative biomarkers. ATR-FTIR probes the first few microns of the sample, which in plants constitutes the external epidermal layers, and therefore provides an overview of the biochemical changes at the plant-environment interface during fruit development. The cutinized cell wall, which forms a biochemically complex heterogeneous matrix as part of the outer epidermis (Yeats and Rose 2013), is composed of various soluble waxes embedded in the main polymer cutin (~40-80%), along with a small phenolic fraction (~1-5%) (Dominguez et al. 2015; Hunt and Baker 1980). The underlying cell wall consists mainly of cellulose, pectin, various polysaccharides, and proteins (Yeats and Rose 2013). During tomato fruit development, the cuticle and cell wall undergo structural and compositional changes that are distinct to the stage of development, including the transition from cell division to cell expansion, cuticle biogenesis, and changes in cell wall thickness (Segado et al. 2016). Consequently, the relative contributions of the cell wall and cuticle to the epidermal plant surface varies markedly during fruit development due to rapid cell division (2 to 35-40 dpa) and the subsequent cell expansion (Azzi et al. 2015). These surface layers therefore present unique *in vivo* molecular targets for distinguishing between developmental stages using ATR-FTIR spectroscopy.

Multi-component analysis over the fingerprint spectrum (1800–900 cm^{-1}) showed that the alterations in these spectral regions were strongly associated with both prominent cuticle and cell wall components including their main constituents. Spectral biomarkers strongly associated with cutin, waxes, and phenolic compounds were all detected (Table 3.1) and are consistent with changes in the cuticle during development 4-36 dpa (Holloway 1982; Hunt and Baker 1980; Yeats et al. 2010). Cutin was identified at wavenumbers 1732, 1725, 1714, 1698, 1692, 1467, 1464, 1173, 1164, 1106, and 1102 cm^{-1}. Waxes, including glycerolipids and suberin-like compounds, were identified at wavenumbers 1732, 1467, and 1464 cm^{-1}. Primary phenolic compounds were identified at wavenumbers 1627, 1558, 1522, 1514, and 1511 cm^{-1}. Other spectral alterations observed originated from cell wall components as part of the cutinized cell wall structure. Spectral biomarkers associated with the cell wall identified cellulose, pectin, and various other carbohydrate moieties. Cellulose and pectin were related to wavenumbers at 1725 1714, 1106,

1102, and 1017 cm^{-1} respectively. Other carbohydrates, including some lignin like compounds, showed overlap with pectin, cellulose, and other moieties at wavenumbers 1522, 1514, 1511, 1164, 1106, and 1102 cm^{-1}. These results show clearly the power of multivariate analysis of fingerprint spectra to provide information about the biochemical changes occurring in the cuticle and cell wall during tomato fruit development. However, further work is needed to decipher the exact role of these compounds in the context of their IR absorptive properties. Importantly, the developmental time frame between 4 and 36 dpa contains at least four horticultural grades used as industry standards; the maturity grades M1-M4 correspond approximately to DS06-DS09 (Kader and Morris 1976; Maul et al. 1998; Sargent 1997). Therefore, the preliminary characterization, qualitative analysis presented here shows the potential for distinguishing developmental stages according to horticultural grades, for example the mentioned M1-M4 grades, based on their epidermal surface properties through the detection of multiple spectral biomarkers related to tomato fruit development. Nevertheless, further investigations are still required to determine the structure-function relationships in tissues at different developmental stages and for horticultural applications such as maturity grading.

3.3.2 Spectral Characteristics of Tomato Fruit Ripening

Ripening, although part of the natural development of tomato fruit, is often seen as a separate developmental stage due to it separate genetic regulation and distinct color changes. Significant shifts in gene expression, and transition in ethylene biosynthesis result in modifications of epidermal surfaces of tomato fruit during ripening, which influence post-harvest qualities (Yeats et al. 2010; Cara and Giovanni 2008; Lara et al. 2014; Azzi et al. 2015). Changes in the epidermal layers of tomato fruit thereby differ significantly from the developmental phase and throughout the ripening period. Figure 3.4 shows that during ripening, tomato fruit exhibit both unique and common spectral features from those observed for development that separate into distinct spectral clusters corresponding to the six ripening stages: mature green, breaker, turning, pink, light red, and red ripe (RS01-RS06, see Figure 3.1).

Distinct biophysical and associated biochemical changes occur in the cuticle and cell wall during tomato ripening approx. 35-55 dpa (Bargel and Neinhuis 2005; Segado et al. 2016). The observed spectral changes in the epidermal layers are therefore likely to be associated with events including cuticle rearrangement, cell wall disassembly, carotenoid accumulation, and the

underpinning changes in genetic and metabolic regulation (Cara and Giavannoni 2008; Yeats and Rose 2013). Consequently, this exploratory analysis clearly shows that biospectroscopy can provide an abundance of chemical information that can contribute to understanding of the changes that occur in the epidermal layers during development and ripening. Importantly, the ability to analyze intact fruit will enable baseline characterizations of the development and ripening of healthy fruit, offering the intriguing possibility of using deviations from the baseline as indicators of abnormal development, stress, or disease. For horticultural applications, ripening is particularly interesting as the late red ripe stage are key stages for consumer consumption but are also stages at which fruit become increasingly susceptible to events such as fruit cracking and pathogen infection, which are linked directly to epidermal structure and fruit integrity (Isaacson et al. 2009; Lara et al. 2014).

3.3.3 Common Spectral Characteristics of Tomato Fruit Development and Ripening

Tomato fruit development and ripening show common spectral features relating primarily to the cuticle and cell wall components. Discriminant wavenumbers common to both fruit development and ripening include the general regions: 1725-1714, 1632-1627, 1561-1558, 1514-1511, and 1473-1464 cm^{-1} (Table 3.2 and 3.3). Specifically, these spectral biomarkers include wavenumbers at 1725, 1721, 1719, and 1714 cm^{-1}, which are strong absorbance contributions of ν(C=O) ester and medium absorbance contribution from ν(C=O· · ·H) ester of cutin. Wavenumbers at 1632 and 1627 cm^{-1} were medium and strong absorbance of ν(C=C) respectively, which were indicative of phenolic compounds. Further weak ν(C-C) absorbance of phenolic compounds was seen at 1561 and1558 cm^{-1}, while regions including 1514 and 1511 cm^{-1} related to ν(C-C)~(C=C) conjugated aromatic entities of phenolic compounds. In the traditional context of IR spectroscopy, these absorbance designations strong, medium, and weak, refer to the highest, intermediate, and smallest peak amplitudes respectively, relative to one another in the spectrum (Stuart 2005); these reflect both the IR activity of functional groups and their abundance in the sample. Common development and ripening spectral biomarkers identified here in intact tomato fruit have also been observed in isolated cuticles of both immature green and red ripe tomato fruit (Heredia-Guerrero et al. 2014). Similarities seen at 1473, 1467, and 1464 cm^{-1} were interpreted as indicating δ(CH_2) scissoring of cutin and waxes present in mature and immature fruit cuticles (España et al. 2014; Heredia-Guerrero et al. 2014). Cutin is one of the outermost and abundant compounds in the cuticle

and therefore the spectral changes observed in both the intact fruit and isolated cuticles are consistent with the changes in cuticular compounds, and especially cutin, during development and ripening. ATR-FTIR analysis as a surface technique identifies common biomarkers in the surface layers of fruit across the total developmental program from ~4-55 dpa, which are associated mainly with changes in the cuticle. However, novel regions in the fingerprint spectrum may provide insights into the exact role of the many cuticle functions in fruit development and ripening (Lara et al. 2014).

3.3.4 Identifying Tomato Fruit Development and Ripening Stages

The ability to distinguish nine distinct developmental stages and six common horticultural ripening grades of tomato fruit autonomously and non-destructively represents an important advance enabling expert growers or industrial food production/supply chains to grade fruit quality more effectively. Sensor-based horticultural systems will rely on multiple inputs from various sensors. For this reason, it is important to explore and employ different sensors and see these as being complementary rather than competitive. Various studies have shown that tomato maturity grading and assessing quality parameters can be achieved using spectroscopies that employ different wavelengths and ranges of the electromagnetic spectrum between UV, visible and IR light. To contribute to the expansion of MIR sensors, it was shown that the present level of accuracy was achieved using a compact spectrometer, relatively small data set compared to the number of samples available for testing in a horticultural setting, where typically classification accuracy increases with larger datasets. Although external validation is necessary to solidify these results, this study provides a clear indication of the potential, specifically ATR-FTIR for automatic classification of various horticultural products including tomato.

3.4 Conclusions

Biospectroscopy is a powerful analytical tool and potential sensor technology for linking fundamental plant biology and applied crop sciences as part of developing precision horticulture systems. The development of surface techniques including MIR spectroscopy that are applicable to both homogenous and for heterogeneous substances has opened the door for analysis of intact tissues and non-destructive measurements *in vivo*. However, to date the degree to which MIR spectroscopy has been used to study intact plants has been limited, as has the evaluation of portable

equipment that may be readily retooled for use in horticultural applications (Farber and Kurouski 2018). The ATR-FTIR sampling mode, probes the main groups of biochemical compounds within tomato fruit epidermal surface layers, such as cutin, wax, and phenolic fractions of the cuticle, as well as cellulose, pectin, carbohydrates, and lignin-like compounds as primary cell wall constituents (Table 3.1 and Table 3.2), and is thus ideal for the study of plant epidermis as it relates to horticultural parameters. Biospectroscopy based multi-compound analysis, within plant organs in vivo, offers an alternative methodology to conventional ways of studying cuticle and cell wall structure during development or in response to industrial processing (Heredia-Guerrero et al. 2014; Largo-Gosens et al. 2014). In this regard, MIR biospectroscopy will prove useful for deciphering the molecular details of changing epidermal structures during tomato fruit development and ripening. This is critical because the detailed mechanisms behind cuticle formation are debated, and little is known about the relationship between cuticle structure and postharvest characteristics in whole tomato fruit (Dominguez et al. 2015; Lara et al. 2014).

As a method for *in vivo* analysis, as demonstrated here on delicate tomato fruit, ATR-FTIR spectroscopy can measure large groups of compounds in epidermal structures of whole tomato fruit. 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. 2016). 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 (Lara et al. 2014). Automatic grading of the defined tomato fruit groups was evaluated using the SVM classification model indicating that development and ripening 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.

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 realize this potential, application of biospectroscopy to additional model plant systems is needed alongside the evaluation of new portable equipment, similar to 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, 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.

3.5 Methods

3.5.1 Plant Growth Conditions

Individual tomato plants, *Solanum lycopersicum* cv. Moneymaker, were grown from commercial seed (Thompson and Morgan Seeds, UK) in 10 L pots containing Levington's M3 growth medium (Levington Horticulture Ltd, Ipswich, UK) to anthesis (approx. 60 days). Plants were grown in a heated glasshouse (25 ± 5 °C) with an 18/6 h day/night cycle (minimum illumination 500 µmol m^{-2} s^{-1} at the plant canopy from 600 W metal-halide lamps) and 50 ± 10% humidity. Tomorite fertilizer (Levington Horticulture Ltd, Ipswich, UK) was applied from anthesis, at every other watering according to the manufacturer's instructions. Criteria for development and ripening stages was dpa, where the initial class was measured at 4 dpa and subsequent classes were separated by 4 days of growth for both the development and ripening series respectively. Tomato fruit parameters used in the selection process were recorded for development and ripening sets corresponding to those shown in Figure 3.1 and are found in Table S3.4 and S3.5.

3.5.2 ATR-FTIR Spectroscopy

Tomato fruit were picked from plants, washed with deionized water, dried and immediately measured using ATR-FTIR spectroscopy. Vibrational spectra were acquired from intact tomato fruit at 9 developmental stages (DS01-DS09) and 6 ripening stages (RS01-RS06). Whole tomato fruit were placed on the sample stage for analysis, with no more than 0.1 kg of applied pressure to ensure adequate sample contact. Five points from each fruit were measured around the circumference; two spectra were taken at each contact point for a total of 10 measurements per fruit. Ten fruits were analyzed, for a total of 100 spectra for each developmental and ripening stage making a total of 900 spectra for the development dataset (9 classes) and 600 spectra for the ripening dataset (6 classes). Spectra were acquired using a compact portable Bruker Alpha-P infrared spectrometer with platinum ATR attachment (Bruker Optics, Coventry, UK), over the range 4000-400 cm^{-1} with a spectral resolution of 8 cm^{-1}, 32 co-additions and a mirror velocity of 7 kHz. Background spectra were taken prior to sample measurement to account for ambient atmospheric conditions. The diamond ATR crystal defined a spatial resolution (sampling area) of 1 mm^2 and was cleaned between measurements with ATR cleaning wipes containing isopropyl-alcohol (Bruker Optics, Coventry, UK).

3.5.3 Computational Analysis

Raw spectra truncated to the spectral fingerprint region (1800–900 cm^{-1}) were pre-processed using the Savitzky-Golay filter and second order differentiation, followed by vector normalization to account for differences in sample thickness and ATR diamond contact pressure. PCA-LDA was used for exploratory data analysis and biomarker extraction. PCA-LDA was performed using the open source IRootlab toolbox (https://github.com/trevisanj/ irootlab) specialized for analysis of IR spectra (Trevisan et al. 2013), in conjunction with Matlab 2016a (The Maths Works, MA, USA). Principal component analysis reduces the dataset down to factors that account for spectral variance; PCA was optimized using IRootlab to ensure the inclusion of the primary dataset variance within the first 10 PCs. The first 10 PCs accounted for more than 97% and 95% of variance in the development and ripening datasets, respectively (Table S3.6). These served as input variables for LDA forming the composite technique PCA-LDA (Trevisan et al. 2012). Exploratory analysis by way of cluster separation along the three main linear discriminants

(LD1, LD2, and LD3) was explored, to determine whether specific clustering of spectral groups, belonging to developmental and ripening stages, could be observed. PCA-LDA scores were cross validated 10 k-folds. For a qualitative characterization of the main spectral alterations, PCA-LDA loadings in combination with a peak-pick algorithm (20 cm^{-1} minimum separation) was used to tabulate the top six most prominent vibrational mode alterations, and their corresponding chemical assignments, which were used as tentative biomarkers for development and ripening (Kelly et al. 2011).

Testing of classification accuracy of DS01-DS09 and separately RS01-RS06 stages with SVM was conducted using the PLS toolbox version 7.9 (Eigenvector Research, Inc., WA, USA); in conjunction with Matlab 2016a. Classification of developmental and ripening stages was performed using an SVM classifier. The SVM classifier was constructed using 90% of data for training and 10% for internal validation. The same data used for PCA-LDA, pre-processed fingerprint spectra, were used as input for SVM. This model was developed to improve on the classification performance of PCA-LDA (Table S3.1-S3.3 and Figures S3.1-S3.2). SVM was cross-validated using 10 k-folds.

3.6 Supplementary Materials

Table S3.1 Predictive performance presented as sensitivity and specificity rates calculated for the PCA-LDA chemometric model intended to differentiate tomato fruit developmental and ripening stages from their ATR-FTIR spectral data.

	Grouped data		**Cross-validation**	
Developmental Stage (dpa)	**Sensitivity**	**Specificity**	**Sensitivity**	**Specificity**
DS01 (04)	100%	100%	100%	100%
DS02 (08)	63%	99%	63%	99%
DS03 (12)	92%	95%	92%	95%
DS04 (16)	85%	100%	84%	100%
DS05 (20)	79%	99%	79%	99%
DS06 (24)	90%	98%	90%	98%
DS07 (28)	69%	98%	69%	98%

DS08 (32)	86%	93%	86%	93%
DS09 (36)	97%	100%	97%	100%
Ripening Stage	**Sensitivity**	**Specificity**	**Sensitivity**	**Specificity**
Mature Green	100%	100%	100%	100%
Breaker	100%	98%	100%	98%
Turning	100%	100%	100%	100%
Pink	90%	100%	89%	100%
Light Red	100%	100%	100%	100%
Red	100%	100%	100%	100%

Table S3.2 Predictive performance presented as sensitivity and specificity rates calculated for the SVM chemometric 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 S3.3 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%

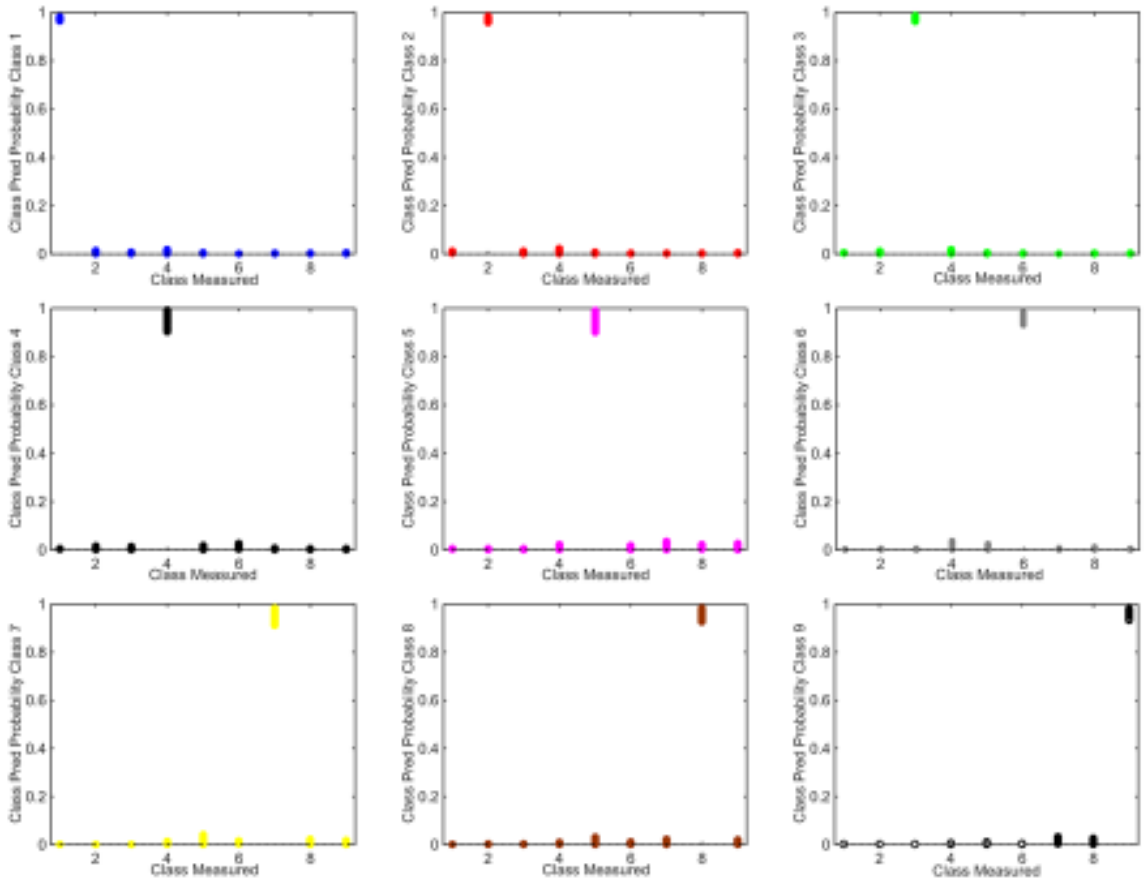

Figure S3.1 Class predictive probability results for individual spectral classes for development (in order DS01-DS09).

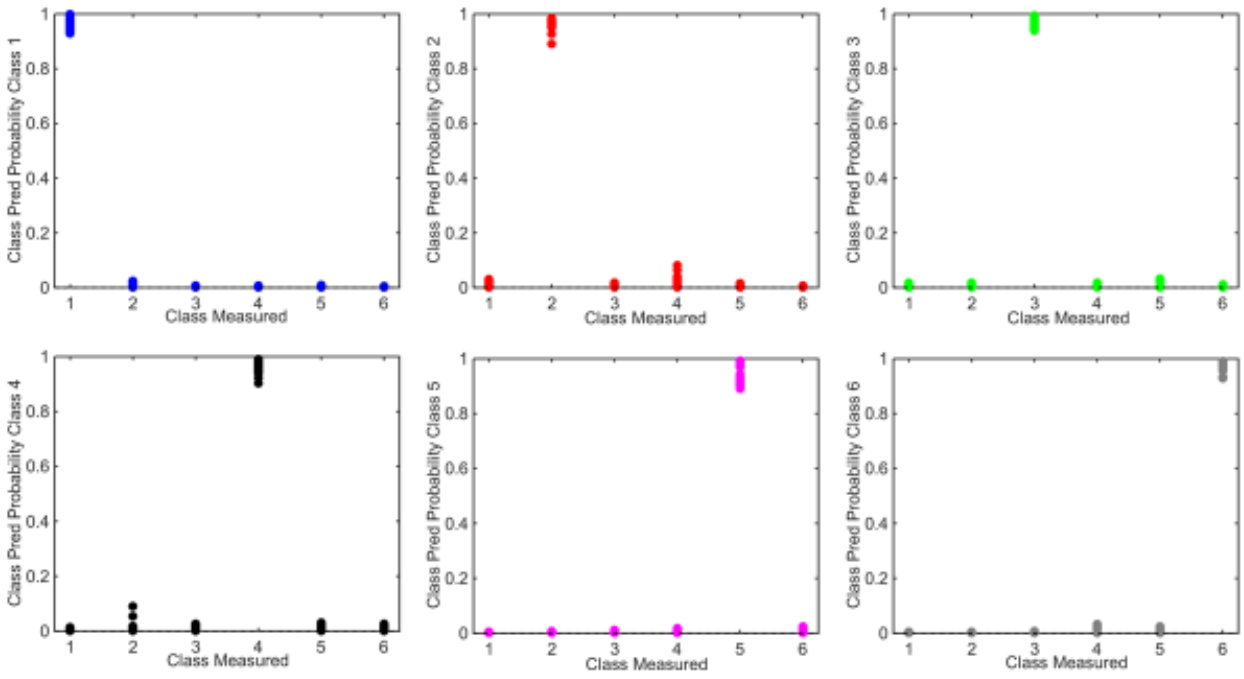

Figure S3.2 Class predictive probability results for individual spectral classes for ripening (in order RS01-RS06).

Table S3.4 Development stages of tomato fruit *Solanum 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 S3.5 Ripening stages of tomato fruit *Solanum 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 color from green to tannish-yellow, pink, or red on not more than 10% of the surface color
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 color
Pink	RS04	30%-60% of the surface is not green; the aggregate, shows pink or red color
Light Red	RS05	60%-90% of the surface is not green; the aggregate shows pinkish-red or red color
Red (Ripe)	RS06	> 90% of the surface is not green; aggregate shows red color

Table S3.6 Percentage of variance for PCA-LDA models varying the number of PCs.

	Developmental Stage (dpa)		Ripening Stage	
PC	Variance (%)	Cumulative Variance (%)	Variance (%)	Cumulative Variance (%)
1	63.80	63.80	49.15	49.15
2	16.77	80.58	37.01	86.15
3	8.77	89.35	3.39	89.54
4	3.60	92.94	1.57	91.12
5	2.34	95.28	1.35	92.47
6	1.00	96.28	0.86	93.33

7	0.62	96.91	0.69	94.02
8	0.49	97.39	0.57	94.59
9	0.32	97.71	0.44	95.03
10	0.25	97.96	0.35	95.38

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Chapter 4: ATR-FTIR Spectroscopy Non-Destructively Detects Damage-Induced Sour Rot Infection in Whole Tomato Fruit

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Abstract

Plant-environment interactions are essential to understanding crop biology, optimizing crop use, and minimizing loss to ensure food security. Damage-induced pathogen infection of delicate fruit crops such as tomato (*Solanum lycopersicum*) are therefore important processes related to crop biology and modern horticulture. Fruit epidermis as a first barrier at the plant-environment interface, is specifically involved in environmental interactions and often shows substantial structural and functional changes in response to unfavorable conditions. Methods available to investigate such systems in their native form however are limited by often required and destructive sample preparation, or scarce amounts of molecular level information. To explore biochemical changes and evaluate diagnostic potential for damage-induced pathogen infection of cherry tomato (cv. Piccolo) both directly and indirectly; mid-infrared (MIR) spectroscopy was applied in combination with exploratory multivariate analysis. ATR-FTIR fingerprint spectra (1800-900 cm^{-1}) of healthy, damaged or sour rot infected tomato fruit were acquired and distinguished using principal component analysis and linear discriminant analysis (PCA-LDA). Main biochemical constituents of healthy tomato fruit epidermis are characterized while multivariate analysis discriminated subtle biochemical changes distinguishing healthy tomato from damaged, early or late sour rot infected tomato indirectly based solely on changes in the fruit epidermis. Sour rot causing agent *Geotrichum candidum* was identified directly *in vivo* and characterized based on spectral features distinct from tomato fruit. Diagnostic potential for indirect pathogen detection based on tomato fruit skin was evaluated using the linear discriminant classifier (PCA-LDC). Exploratory and diagnostic analysis of ATR-FTIR spectra offers biological insights and detection potential for intact plant-pathogen systems as they are found in horticultural industries.

Key words: MIR spectroscopy, plant-pathogen interaction, multivariate analysis, crop biology, *Geotrichum candidum*

4.1 Introduction

Providing food security for a rapidly growing global population of which a large fraction is malnourished is one of the greatest challenges in the modern era (IFPRI 2017). Conventional solutions such as increased land clearing and increasing usage of pesticides to produce sufficient food are unfavorable due to their environmental impacts and long-term unsustainability. Thus, novel alternatives are needed to efficiently produce the approximately 70% more food needed by 2050 (Beuchelt and Virchow 2012). Crop loss to pests and pathogens throughout food production/supply represent a major threat to this aim. Pests and pathogens may reduce crop yield by 80%, thereby presenting a significant challenge to crop productivity (Oerke 2006). Detection of pests and pathogens within pre- and post-harvest settings is therefore essential to minimize the impacts on crop production. The post-harvest consumer stage can be viewed as one of the most critical points during food production/supply, because maximum resource allocation has occurred at this point, making plant-pathogen interactions especially relevant at this stage. Current methods for diagnosing crop health include remote sensing, molecular-based methods and complex analytical techniques all of which have drawbacks. Remote sensing, including hyperspectral imaging and thermography, are highly responsive to environmental conditions and distance to the measured object making it difficult to determine disease specificity (Mahlein 2016). Molecular based techniques (for example, polymerase chain reaction (PCR), fluorescent *in situ* hybridization (FISH), and enzyme-linked immunosorbent assay (ELISA) are time consuming and prone to contamination (Chitarra and Van Den Bulk 2003; Schaad and Frederick 2002; Wallner et al. 1993). Complex analytical methods, for example gas or liquid chromatography coupled to mass spectrometry (GC/LC-MS), require extensive sample preparation and are difficult to use in the field (Martinelli et al. 2015).

The development of flexible, non-destructive sensors capable of providing adequate detection sensitivity and pathogen specificity are keys goal for the detection of crop pathogens (Mahlein 2016; Skolik et al. 2018). This includes the ability to detect early effects of plant stress or disease, to differentiate between the effects of abiotic and biotic stresses and between different diseases, and to quantify the severity of the stress or disease (Mahlein 2016). While various molecular and imaging techniques to detect crop pathogens are under development, the limitations of many analytical methods combined with the above criteria for sensor technologies, has led to

the development of label-free, non-destructive spectroscopic techniques that provide information about the chemical structure of analysed samples. The spectroscopic approach, originating in analytical chemistry, has been translated to the biological sciences mainly through advancements in computational analysis and the ability to measure live samples (Chan and Kazarian 2016). Application of these techniques to model plant and crop systems has the potential to both provide novel insight into plant-pathogen interactions, whilst generating a large number of variables for autonomous classification of disease states for detection of pests and pathogens.

Mid-infrared (MIR) spectroscopy has made substantial headway in the biological sciences as a non-destructive and rapid bioanalytical sensor technology (Martin et al. 2010). This is because MIR spectra have been effective at providing molecular insights into biological systems, while providing a large number of variables on which to discriminate samples. More recently, spectrochemical techniques have made substantial progress in the plant and crop sciences, specifically with regard to the analysis of dynamic processes and plant biology-related to crop production (Butler et al. 2015; Butler et al. 2017; Ord et al. 2016; Skolik et al. 2018). This has been effective mainly through the development of new data analysis methods including multivariate analysis. For MIR biospectroscopy, data analysis can be split into two main types: exploratory and diagnostic. Exploratory analysis is aimed primarily at data visualization and pattern recognition (Trevisan et al. 2012). Diagnostic analysis employs the use of classifier algorithms to evaluate the potential for diagnosis of sample condition (healthy *versus* diseased for example) (Trevisan et al. 2012). While the two frameworks typically have different objectives, they are closely linked and in general, the exploratory precedes the diagnostic framework. Among the many available data analysis options, unsupervised principal component analysis (PCA) and supervised linear discriminant analysis (LDA) have been used alone or in combination to successfully investigate a large number of biological phenomena based on MIR data (Li et al. 2015; Strong et al. 2017). Both PCA and LDA have formed core components of biospectroscopy data analysis. Related classifiers including support vector machine (SVM) and linear discriminant classifier (LDC) have also found ample application in the diagnostic framework. Such advancements have highlighted the potential for MIR biospectroscopy as an effective sensor technology for the plant and crop sciences (Skolik et al. 2018). Despite this progress, the number of investigations on intact samples has been limited, which is arguably an important prerequisite for the development of fully non-destructive horticultural sensors, and thus more research on intact

samples is required. Recently, both attenuated total reflection Fourier-transform infrared (ATR-FTIR) and Raman spectroscopy have been favored for studying intact samples of important crops (Butler et al. 2015; Ord et al. 2016; Fu et al. 2016; Trebolazabala et al. 2013). Raman spectroscopy is a complementary method to FTIR spectroscopy and the two are often combined for a more robust analysis, as each have specific drawbacks due to the distinct light-matter interactions they measure (Baker et al. 2014; Butler et al. 2016). Compared to macro-FTIR, Raman scattering as a low probability event can be highly variable, prone to interference from fluorescence, featuring a small measurement area typically between 20-30 μm, and uses more intense laser powers potentially leading to photobleaching (tissue decomposition) of delicate organic samples (Butler et al. 2016; Yeturu et al. 2016). Nevertheless, a strong case has been made by previous experiments demonstrating the effectiveness of Raman spectroscopy for direct detection of microbial pathogens in intact crops (Egging et al. 2017; Faber and Kurouski 2018; Yeturu et al. 2016). While direct detection of a plant-pathogen interaction generates spectral changes suitable for disease detection, indirect detection of plant infection through spectral changes in tissues because of pathogen attack remains difficult but offers a novel approach especially for early or pre-symptomatic stages of disease (Skolik et al. 2018). ATR-FTIR has the advantage of macro-measurements increasing the measurement area while also affording a very defined magnitude of light penetration into the sample (Kazarian and Chan 2013; Chan and Kazarian 2016) This may be more suitable for analysis at the whole-plant level as many experiments still rely on previous removal (cutting) of leaves and fruit, which is not truly non-destructive (Trebolazabala et al. 2017; Yeturu et al. 2016). It is therefore essential to evaluate Raman complementary methods such as FTIR, as a combination approach can overcome the limitations of a single technique, as well as the variable nature of crops and plant-pathogen systems covered by modern agriculture.

Here we use ATR-FTIR spectroscopy to study the effects of damage and ambient infection by the sour rot causing agent *G. candidum*, both directly and indirectly, in commercially obtained, consumer-stage (red ripe) intact cherry tomatoes. Conventional spectral analysis for the characterization of main absorbance peaks of tomato and fungus *G. candidum* is followed by exploratory and diagnostic multivariate analysis to probe subtle biochemical changes induced indirectly by damage and infection. Changes to the surface of tomato fruit are characterized in response to damage and sour rot infection using the tandem technique of PCA-LDA to maximize inter-class differences between damage, infected, and control fruit. The diagnostic potential of this

approach is evaluated using the tandem classifier PCA-LDC, to distinguish damaged and infected tomato fruit from healthy controls indirectly and autonomously.

4.2 Materials and Methods

4.2.1 Sample Preparation and Storage

Vine cherry tomatoes cv. Piccolo were obtained from a local supermarket (Sainsbury's Lancaster Main Store, UK). All analyses were performed prior to the advertised expiration date, which at the time of purchase presented a window of 8 days. Tomatoes were removed from their commercial packaging taken off the vine and adapted to room temperature (23 ±1ºC) and 35-40% relative humidity for 2 h prior to initial analysis. Loose tomatoes were split into two sets, a control series accounting for changes occurring in naturally ripening tomatoes over the analysis timeframe (Figure 4.1a-c). As well as a set of tomatoes punctured through the stem scar, at 0 h, to a depth of approximately 1 cm with a 21-gauge sterile syringe needle leaving the remainder of the skin intact (Figure 4.1d). Damaged tomatoes were thus susceptible to ambient infection at the puncture site, which was visible starting at 48 h post puncture, and full colonization of the punctured stem scar was observed at 96 h post puncture (Figure 4.1f). Control and damaged tomatoes were dark stored in cardboard boxes under identical conditions to compare pathogen development and allow stem scar infection with ambient fungal spores. Damaged tomato fruit (0 h) were subsequently analyzed opposite their shelf-life matched controls, allowed to age naturally, at 48 and 96 h post puncture, to assess pathogen infection caused by initial damage. Prior to analysis, tomato fruit were washed thoroughly with de-ionized water to remove dust and debris, as well as fungal growth and fluid exudate present on fruits at the early infection (48 h) and late infection (96 h) stages, prior to spectral acquisition, in order to characterize changes in fruit skin, without contribution from the fungus itself (analyzed separately) or exudates from the site of infection. The fungal-fruit complex on fully colonized tomatoes at 96 h post puncture (Figure 4.1f) was analyzed to obtain spectra from the fungus in its native state on tomato fruit.

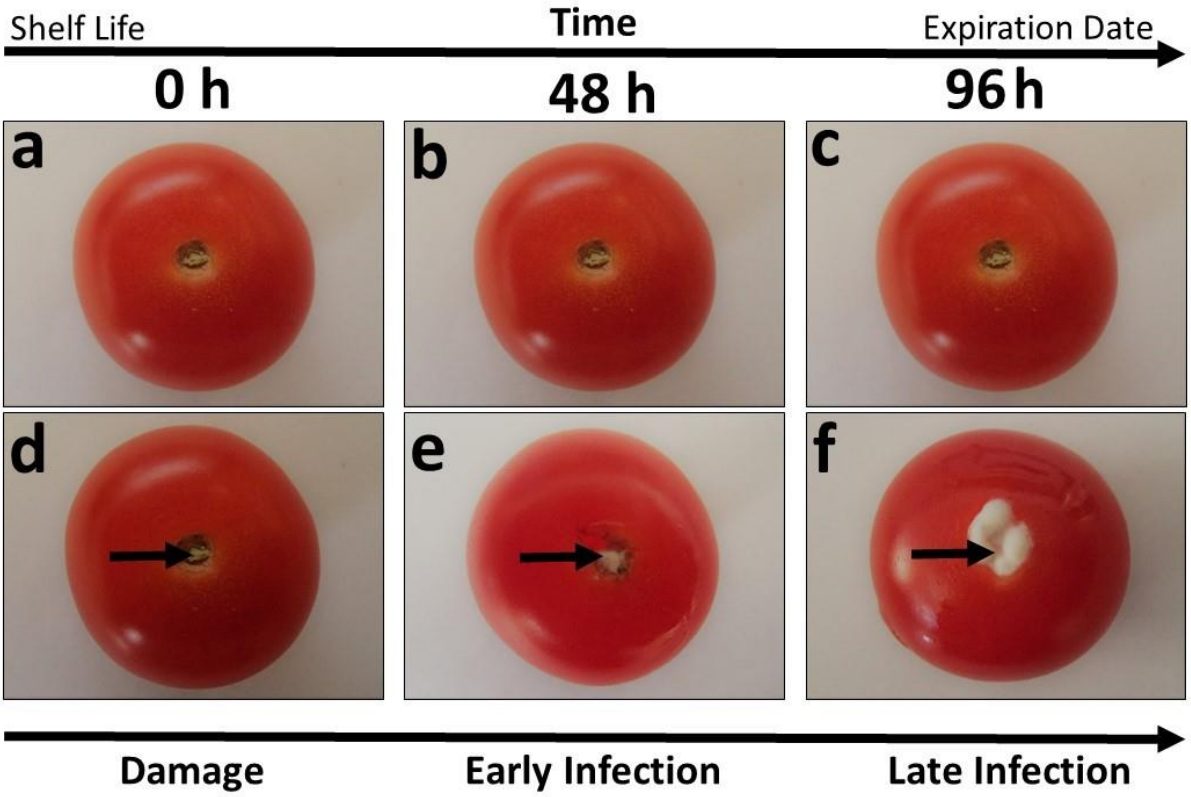

Figure 4.1 Symptoms associated with tomato fruit damage (d), early (e), and late (f) infection of tomato fruit by *G. candidum* compared to their age-matched controls (a-c).

4.2.2 ATR-FTIR Spectroscopy

MIR spectra were acquired from intact tomato fruit, using a Bruker Tensor 27 IR spectrometer with Diamond ATR Helios attachment (Bruker Optics, Coventry, UK). Spectra were acquired over the range 4000-400 cm^{-1} with a spectral resolution of 8 cm^{-1}, 3.84 cm^{-1} data spacing, 32 co-additions and a mirror velocity of 2.2 kHz for optimum signal to noise ratio (Martin et al. 2010; Baker et al. 2014). Background spectra were taken prior to each sample to account for ambient atmospheric conditions. The diamond ATR crystal defined a spatial resolution (sampling area) of approximately 250 µm × 250 µm. Whole fruit were placed on the sample stage with no more than 0.1 kg of applied pressure. Between sample measurements, ATR cleaning wipes containing isopropyl-alcohol (Bruker Optics, Coventry, UK) were used to clean the ATR diamond crystal between measurements. Five points from around the fruit circumference were measured, two spectra from each of the 5 points for a total of 10 measurements per fruit. Ten (10) spectra per fruit generally supply enough replicates for PCA-LDA analysis, which provide intra-class

differences (i.e. variance specific to 0 h control vs 0 h damage, 48 h control vs 48 h early infection, and 96 h control vs 96 h late infection) while minimizing the effect of natural tissue heterogeneity potentially masking the subtle effects underpinning plant response to pathogen. Six fruits were measured for each treatment group. Measurements of *G. candidum* were taken *in vivo* without modification as part of the tomato-pathogen complex at late infection state (96 h post-puncture). The fungal mass completely covered the ATR crystal during measurements; six separate samples (10 spectra from each fungal sample) of *G. candidum* were measured to obtain a representative *in vivo* spectrum.

4.2.3 Pre-Processing and Computational Analysis

All computational analysis was conducted using the open source IRootlab toolbox (https://github.com/trevisanj/irootlab) specialized for analysis of IR spectra (Trevisan et al. 2013), in conjunction with Matlab 2016 (The Math Works, MA, USA), unless otherwise stated. Raw spectra were truncated to the spectral fingerprint region between 1800–900 cm^{-1}, which is the primary region where biomolecules absorb IR radiation. Fingerprint spectra were pre-processed using the Rubber band-like baseline correction algorithm and maximum normalized to account for differences in sample thickness and ATR diamond contact pressure. Class mean spectra were used for direct analysis. Exploratory PCA reduces the dataset down to factors that account for spectral variance; PCA was optimized using the IRootlab *pareto* function, where the first 10 PCs accounted for more than 99% of the variance in the dataset [see Supporting Information (SI) Figure S4.1]. These served as input variables for LDA forming the composite technique PCA-LDA (Trevisan et al. 2012). While PCA reduces the complexity of the spectral data, it is unsupervised, does not account for class labels, views all classes as one and therefore does not distinguish between control, damaged, or infected tomatoes for the purposes of extracting class specific differences (Trevisan et al. 2012). Combined with a supervised approach, LDA following PCA (PCA-LDA), maximizes the spectral differences between classes (control vs damage/infected), and thus allows the extraction of the class-specific biomarkers associated with damage and subsequent sour rot (Martin et al. 2010; Kelly et al. 2011; Trevisan et al. 2012). Pairwise comparisons between two classes generate one linear discriminant (LD), which summarizes the main class-specific differences between control and afflicted tomato fruit. This linear discriminant can be visualized as cluster plots, where each spectrum is defined as a point where overlap and separation of points indicate

similar or dissimilar features respectively (Trevisan et al. 2012). PCA-LDA loadings provide a 'spectrum-like' graph indicating the wavenumbers at which variance between classes is most pronounced, as indicated by the peak magnitude (variance). Peak maxima are used as 'spectral biomarkers' indicative of the biological process under investigation (Kelly et al. 2011).

Exploratory analysis by way of cluster separation along LD1 was explored, to determine whether significant alterations between control and damaged/infected groups were evident. In each case, a pairwise comparison conducted of damaged, early, and late infected tomatoes at 0, 48, and 96 h, with their shelf-life matched controls. For a characterization of the main spectral alterations, PCA-LDA loadings, in combination with a peak-pick algorithm (20 cm^{-1} minimum separation) identifying peak maxima, was used to tabulate the top six most prominent wavenumber alterations (spectral biomarkers). Identified spectral biomarkers were given chemical assignments matched to previously characterized spectral biomarkers, considering parameters including species, tissue type, instrumentation (method, measurement area, interrogation depth, data analysis), and biological interaction (plant-pathogen).

Group classification was evaluated using PCA in combination with a linear discriminant classifier (PCA-LDC), which tests autonomous classification accuracy based on spectral differences (Butler et al. 2017; Gajjar et al. 2013; Friedman et al. 2001). PCA-LDA and PCA-LDC were cross-validated using 10 k-folds. Further information regarding analysis of biospectroscopy data can be found at (https://github.com/trevisanj/irootlab) and in the literature (Trevisan et al. 2012; Kelly et al. 2011).

To test for statistically significant differences in PCA-LDA scores along the primary LD between controls, damaged, and infected tomato, LD1 scores for each biological sample were averaged and tested for significance using unpaired two-tailed *t*-tests (Graphpad, Prism 2014).

4.3 Results and Discussion

4.3.1 Spectral Characterization of Surface Structures of Intact Tomato Fruit *Solanum lycopersicum*

Spectra from whole tomato fruit surface structures reflect prominent biochemical components present in the cuticle and cell wall. There were no differences visible in the appearance of control, undamaged tomato fruit during the 96 h analysis window (Figure 4.1a-c). In contrast,

damaged tomato (0 h) had a small puncture wound from the syringe at which fungal infection developed (Figure 4.1d-f). Figure 4.2 shows the primary absorbance intensities of intact tomato fruit corresponding to Figure 4.1; for the control set at 0, 48, and 96 h (Figure 4.2a) and damaged (0 h), early infected (48 h), and late infected (96 h) (Figure 4.2b) over the baseline corrected and normalized ATR-FTIR fingerprint spectrum over the region (1800-900 cm^{-1}). Comparison of spectra from both control and damage/infected classes shows that the top six main vibrational bands, and chemical assignments, were identical as depicted in Figure 4.2 and Table 4.1. Absorbance intensities shown in Figure 4.2 and assigned in Table 4.1 reflect prominent biochemical components of plant surface structures including cutin, phenolic compounds, waxes, and potentially volatile organic chemicals (VOCs) (Baldassarre et al. 2015). Several of these compounds had been identified previously from the inner or outer face of isolated tomato cuticles (España et al. 2014; Heredia-Guerrero et al. 2014); despite differences in spectral resolution and equipment used to characterize isolated tomato cuticles (España et al. 2014). This is consistent with the thickness of the cuticle during the late red ripe stage of tomato fruit (España et al. 2014; Heredia-Guerrero et al. 2014) and the shallow interrogation depth of the ATR-FTIR beam (~1-3μm). Cuticle components readily identified include vibrational modes associated with the main polymer cutin at wavenumbers 1728, 1462, 1165, and 1103 cm^{-1} (España et al. 2014; Heredia-Guerrero et al. 2014). Phenolic compounds are among other cuticle constituents that strongly absorb IR radiation, and were identified by absorption at 1605 cm^{-1} (España et al. 2014; Heredia-Guerrero et al. 2014). We also identified an absorption peak at 1223 cm^{-1} that is not present in isolated cuticle (Heredia-Guerrero et al. 2014) but which has been previously associated with monoterpenes, more specifically geranyl-acetate, a structural component of many VOCs (Ord et al. 2016; Rodríguez et al. 2013; Schulz and Baranska 2007). Tomato fruit produce a characteristic profile of secondary metabolites including VOCs during ripening (Petro-Turza, 1986; Buttery et al. 1987, 1990) and it is likely that monoterpenes characteristic of VOCs present at the red-ripe stage, represent a unique contribution to the fingerprint spectrum of intact tomato fruit compared to isolated cuticle (Rodríguez et al. 2013). Alternatively, it is possible that the absorption at 1223 cm^{-1} may simply be a broad absorption band related to the previously identified δ(OH) mode between 1246-1243 cm^{-1} associated with both cutin and polysaccharides (Heredia-Guerrero et al. 2014). Both cuticle and underlying plant layers including the cell wall have been well studied using MIR based biospectroscopy (Heredia-Guerrero et al. 2014; Largo-Gosens et al. 2014). However,

due to several caveats, the number of studies on intact, and hence physiologically competent, samples have been limited, limiting also the development of vibrational spectroscopy for applied horticulture (Skolik et al. 2018). Characterizing spectral features of tomato fruit *in vivo*, such as the cuticle, provides a first but important step in this endeavor and will contribute significantly to the sustainability of crop protection measures. Yet the role of the cuticle, and other epidermal structures as part of the tomato fruit skin, in post-harvest quality, shelf-life, and pathogen susceptibility remains debated especially at the molecular level, in part due to the difficult nature of this recalcitrant layer and the intimate relationship with the underlying cell wall (Dominguez et al. 2015; Lara et al. 2014). To shed light on this, analytical surface techniques such as ATR-FTIR spectroscopy are ideal, as demonstrated by the ability to measure delicate intact fruit truly non-destructively *in vivo* using ATR-FTIR (Figure 4.2). But before the molecular *in vivo* details can be uncovered, surface characterization of intact fruit is necessary to aid the interpretation of more subtle changes hidden in the spectral data, which can only be extracted through multivariate analysis, similar to how previous cuticle component characterization aids the interpretation of the tomato fruit skin *in vivo* shown in Figure 4.2 (Heredia-Guerrero et al. 2014). Comparing between isolated constituents and their native arrangements, in fruit or otherwise, will remain necessary to aid in the identification of candidate target compounds to serve as spectral biomarkers for varying conditions, especially dynamic physiologically driven ones, including plant-pathogen interactions. It is therefore important to characterize the candidate plant compounds being measured by biospectroscopy techniques, for appropriate interpretation of spectral data from physiologically competent samples *in vivo*. Further, indirect detection of damage to the fruit surface (cuticle, cell wall, epidermis) and pathogens affecting crops, such as tomato which are easily compromised by damage leading to infection, would be of utmost interest for commercial development. Once characterized, changes in the MIR signature caused by abnormalities such as damage, pathogen infection, or stress, will prove useful for monitoring fruit condition as it pertains to shelf life through the post-harvest food system, thereby improving crop utilization.

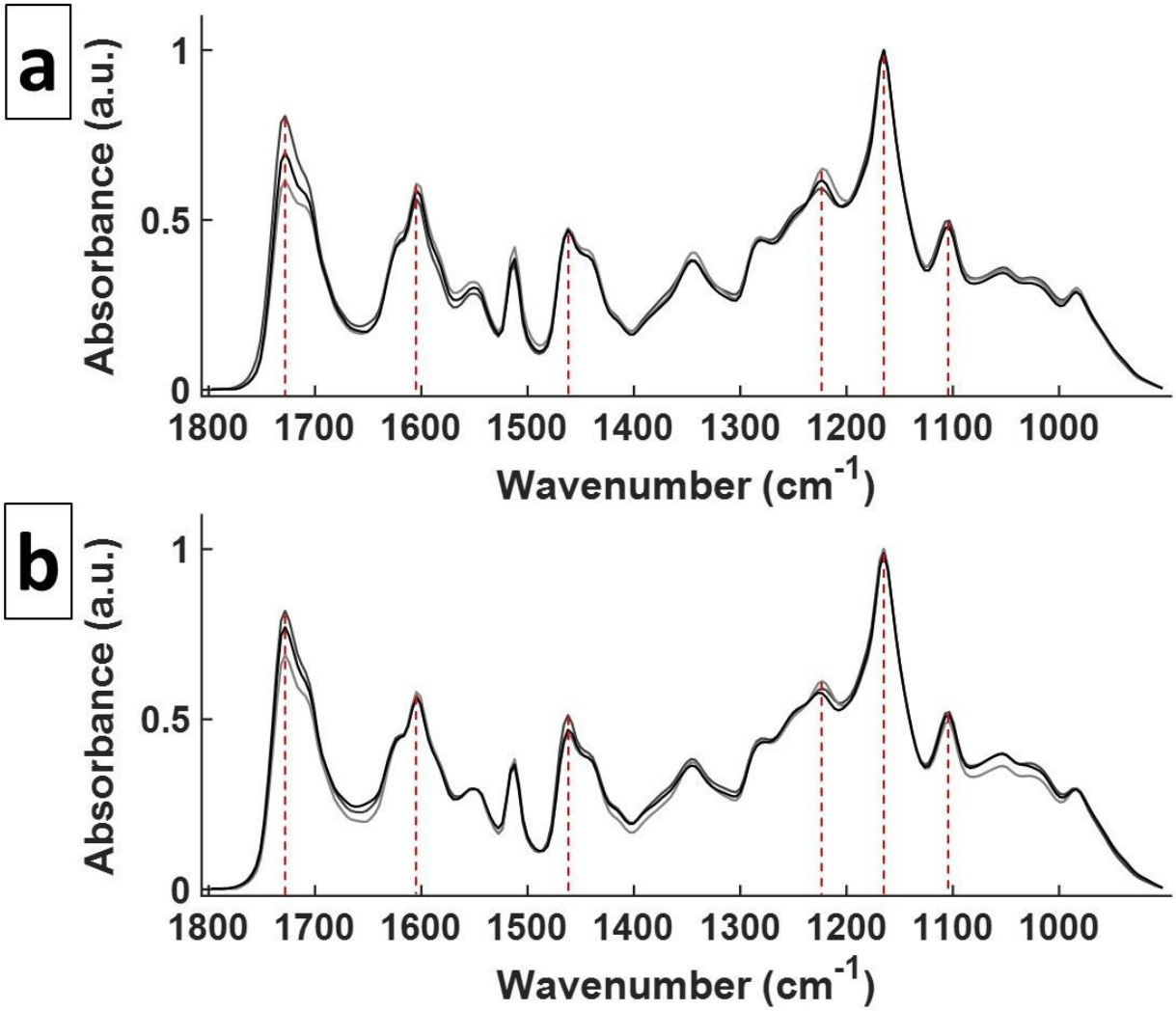

Figure 4.2 ATR-FTIR spectrum of intact tomato fruit *S. Lycopersicum* cv. Piccolo, over the fingerprint region (1800-900 cm^{-1}); **a** control series at 0 (light grey), 48 (grey), and 96 (black) h; **b** 0 h damaged (light grey), 48 h early infection (grey), and 96 h late infection (black).

Table 4.1 Primary absorbance peaks of intact tomato fruit *S. Lycopersicum* cv. Piccolo

Wavenumber (cm^{-1})	Vibrational Mode	Biochemical Assignment	References
1728	ν(C=O) ester	Cutin	[1], [2], [3]
1605	ν(C-C) aromatic	Phenolic compounds, Pectin	[1], [2], [4]
1462	$\delta(CH_2)$ scissoring	Cutin and other waxes, Polysaccharides	[1], [2], [4]
1223	ν_a(C-O-C) ester	Monoterpene	[2], [3], [4]
1165	ν_a(C-O-C) ester	Cutin, Cellulose, Pectin	[1], [2], [3], [4]
1103	ν_s(C-O-C) ester	Cutin, Cellulose, Pectin	[1], [2], [3], [4]

[1] Heredia-Guerrero et al. 2014; [2] Movasaghi et al. 2008; [3] Ord et al. 2016; [4] Schulz and Baranska 2007

4.3.2 Spectral Alterations Associated with Tomato Fruit Damage and Sour Rot Infection by *Geotrichum candidum*

The MIR spectrum of fruit surface structures is altered in response to damage through the stem scar and subsequent infection by *G. candidum.* Artificially damaged tomatoes (Figure 4.1b), exposed to ambient conditions showed no initial signs of fungal infection after 24 h (data not shown), whilst at 48 h post-puncture (early infection) clear signs of infection were evident around the puncture site (black arrows) (Figure 4.1d), and at 96 h post-puncture (late infection) substantial pathogen growth had covered the puncture site (Figure 4.1f). Based on visible symptoms starting at 48 h post puncture (Figure 4.1d, f) the pathogen was determined to be *G. candidum,* a non-specific fungus known as a ubiquitous contaminant of tomato processing equipment (Thornton et al. 2010). Because the mean spectra of control and damage/infected tomatoes (Figure 4.2a and 4.2b) were nearly identical, with respect to direct comparison of main vibrational bands (Figure 4.2 and Table 4.1), PCA-LDA was employed to investigate if class-specific effects were detectable between control and compromised tomatoes. This approach was intended to determine if any changes caused by damage and pathogen infection were observable indirectly without contributions from the fungus itself.

Class specific differences for damage, early, and late infection were observed for tomato fruit compared to their healthy counterparts, as determined by multivariate analysis using PCA and LDA in tandem (PCA-LDA). Pairwise comparisons (class versus control) lead to the generation of a single LD, in this case generating three PCA-LDA scores plots (Figure 4.3a-c). Figure 4.3 shows a clear separation of clusters belonging to each paired class, indicating differences in spectra acquired from controls and damaged, early, and late infected (Figure 4.3a, b, and c respectively). Separation along LD1 indicates significant differences within fingerprint spectra, which are specific to damage and infection. PCA-LDA scores plots reveal significant data cluster separation, with statistical differences between damaged ($p=0.003$), early infected ($p=0.0001$), and late infected ($p=0.0003$) fruit, compared to their shelf-life matched controls. This suggests that spectral changes are most pronounced for the early infected stage, showing the largest degree of separation along LD1 (Figure 4.3b), followed by the late infected stage (Figure 4.3c) and damaged fruit (Figure 4.3a) respectively. Loadings plots (Figure 4.3d-f) indicate the wavenumber regions responsible for the observed cluster separation within the PCA-LDA scores plots (Figure 4.3a-c) (Martin et al. 2007; Trevisan et al. 2012). Wavenumbers identified through peaks within loading pots, represent the areas with the highest degree of variance. Table 4.2 summarizes the top six discriminating wavenumbers identified from PCA-LDA loadings plots. These top six wavenumbers, identified via the peak picking algorithm described, are assigned as tentative spectral biomarkers responsible for the class-specific differences. Spectral biomarkers identified by PCA-LDA were considered a match if these were within ±10 wavenumbers of those identified within the other classes. It is noteworthy that because PCA-LDA potentially extracts very subtle differences within complex tissue architectures, biomarkers identified this way may not originate from the prominent cuticle components evident in the fingerprint spectra shown in Figure 4.2 but may represent small fractions of molecules embedded in the epidermal matrix. For this reason and without extensive validation of their origin, biomarkers are assigned tentatively.

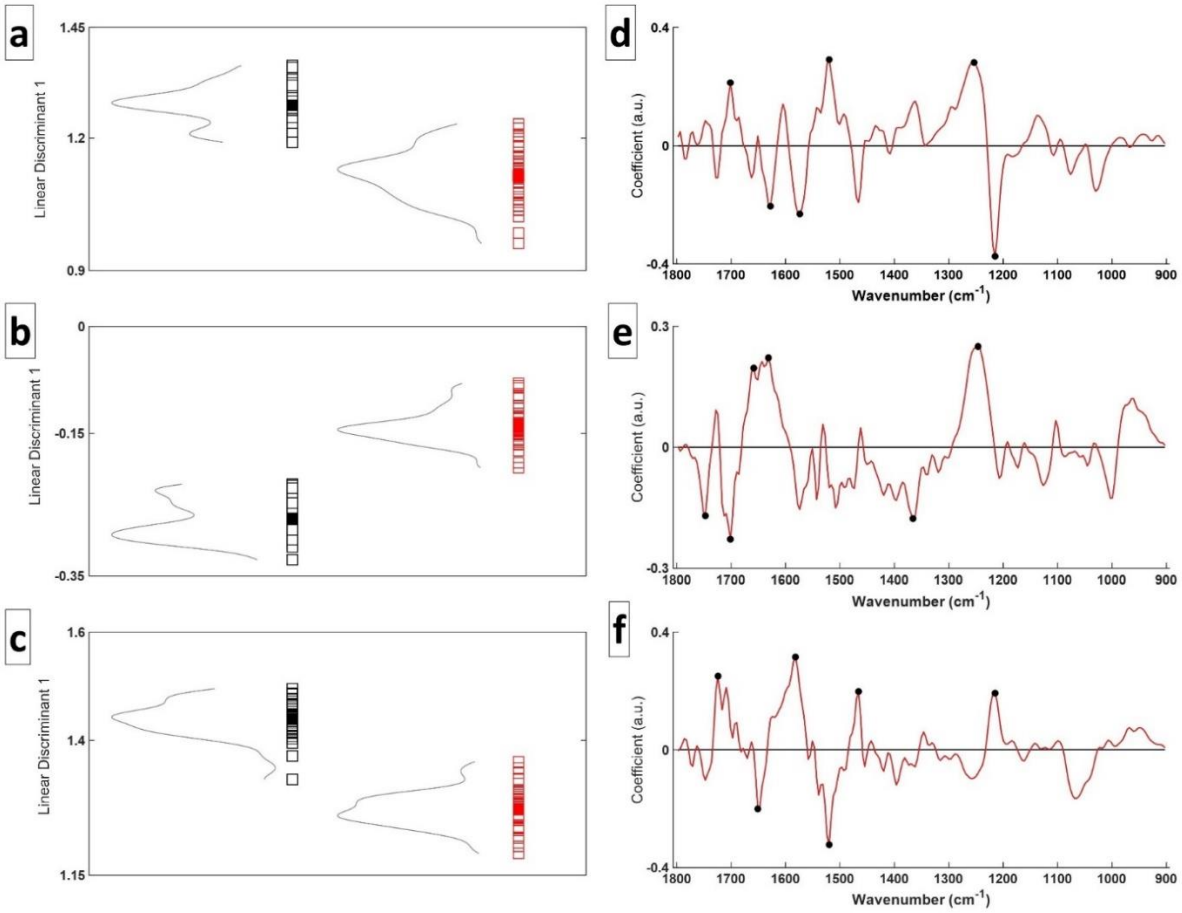

Figure 4.3 PCA-LDA 1-dimensional scores plots showing class specific cluster separation indicative of spectral differences between damaged, early, and late infection opposite their shelf-life matched controls (a-c); corresponding loadings show specific wavenumbers responsible for clustering along LD1 (d-f).

Several wavenumbers identified for the various fruit conditions showed overlap, where biomarkers as discriminators for initial damage were also identified as discriminators for early and late infection. Vibrational modes at 1701, 1632-1628, 1254-1246 cm^{-1} were seen to be consistent between initial fruit damage and early *G. candidum* infection. Absorption at 1701 cm^{-1} was the only exact match between these two classes. These three wavenumber regions are assigned as carbonyl groups in fatty acid esters of cutin (1701 cm^{-1}) (España et al. 2014); carbon-carbon bonds in phenolic cuticular compounds (1632-1628 cm^{-1}) (Heredia-Guerrero et al. 2014); and hydroxyl group deformation in cutin or other polysaccharides (1254-1246 cm^{-1}) (Heredia-Guerrero et al. 2014), which are part of the epidermal surface. Alternatively, the region from 1254-1246 cm^{-1} has

been associated with the Amide III band of proteins or methylene functional groups of phospholipids (Mogashavi et al. 2008), which are also potential targets of ATR-FTIR as part of the epidermis. Consistency within wavenumbers was also observed between those indicative of damage and those identified within late stage *G. candidum* infection, specifically absorption bands at 1582-1574, 1520, and 1215 cm^{-1}. Interestingly, both absorption at 1520 and 1215 cm^{-1} were exact matches to wavenumbers related to initial fruit damage and may therefore play a role in both damage response and response to pathogens (Table 2). Absorption bands between 1582-1574 cm^{-1} are strongly associated with the amide II band of proteins (Mogashavi et al. 2008). The absorption band at 1520 cm^{-1} is potentially a shoulder region of the amide II peak but more likely associated with carbon-carbon bonds in phenolic compounds (Heredia-Guerrero et al. 2014), although this region has also been associated with alkene groups in aromatic compounds, or the imine group in nucleic acids (Mogashavi et al. 2008). Class unique wavenumbers occur only in the early and late infection stages (upon appearance of visual symptoms). All absorption bands identified in damaged tomato occur also in either early or late infection and generate no unique absorbance peaks within the top six tentative biomarkers. Wavenumbers unique to early infection include absorbance at 1747 and 1366 cm^{-1} (Table 2). Vibrational modes at 1747 cm^{-1} are associated with double bonds in carbonyl and alkene functional groups of cutin, wax and suberin-like compounds, as well as lipids in general (España et al. 2014; Heredia-Guerrero et al. 2014). Besides compounds including cutin and waxes, also cellulose, pectin, polysaccharides, and sesquiterpenes are biomolecules which have vibrational modes that absorb at 1366 cm^{-1} (Largo-Gosens et al. 2014; Heredia-Guerrero et al. 2014; Mogashavi et al. 2008). These spectral biomarkers appear to be unique to early infection of tomato fruit (Table 2). In contrast, late infection of tomato fruit shows specific absorbance at 1724 and 1466 cm^{-1} and are associated with carbonyl vibration of cutin, lipids, polysaccharides, or phenolic esters; and methylene vibration of cutin or other waxes respectively (Heredia-Guerrero et al. 2014; Mogashavi et al. 2008). Taken together, these results indicate prominent changes occurring simultaneously across several compounds including lipids, proteins, and carbohydrates, many of which represent prominent components of the epidermal structure including cuticle and cell wall components.

Spectral alterations associated with tomato fruit damage are partially retained during subsequent early and late pathogen infection. Initially, tomato damage induces a wounding response, as colonization by *G. candidum* has not yet occurred (Figure 4.1d; Figure 4.3a, d),

suggesting that the observed spectral alterations are specific to wounding, entailing a stress response. Both metabolic activity and VOC composition change in response to plant wounding; at the red ripe stage, damage elicits changes in the VOC profile (Baldassarre et al. 2015). Wavenumbers identified as discriminators for fruit damage may therefore reflect prominent changes to the VOC profile, potentially combined with upregulation of genes involved in defense reactions and the resulting changes in metabolism (Baldassarre et al. 2015). As VOCs diffuse through plant surface layers, their interaction with the cuticle, cell wall, or epidermis in general may produce alterations in these layers leading to the observed spectral changes (Penuelas and Llusia 2001). As damage has a direct effect on post-harvest deterioration and shelf life through various biochemical and physiological events (Watada and Qui 1999), rapid damage detection of tomato fruit using spectrochemical analysis would help prevent subsequent infection and spoilage induced by spreading microorganisms such as *G. candidum.* Spectral biomarkers from the initial response to wounding are retained in part during subsequent early and late infection (Table 4.2). Although several spectral biomarkers are consistent between damaged and early as well as late infected tomatoes, both early and late infection show unique spectral characteristics as well. It therefore seems plausible that changes in tomato fruit surfaces resulting from damage, share common biochemical alterations with early and late stage infection for example through a general stress response transitioning into a pathogen specific response, explaining the overlap in biomarkers previously described (Table 4.2). As an increasing number of genetic and metabolic changes are induced by wounding and subsequent infection, the change in spectral profile likely reflects the move from damage response to plant-pathogen interaction, explaining the development of unique biomarkers at the early and late infection stages (Table 4.2).

Table 4.2 Top six discriminating class-specific wavenumbers and tentative chemical assignments, from LD1 loadings plots associated with tomato fruit damage, early infection or late infection *versus* control classes.

Class	Wavenumber (cm^{-1})	Vibrational Mode	Biochemical Assignment	References
Damaged	1701 *	ν(C=O· · ·H)	Fatty acid esters Cutin	[3], [6]
	1628 *	ν(C=C) ν_s(C-C) ring Amide I	Phenolic compounds Pectin Proteins	[1], [2], [3], [4], [6]
	1574 ^	Amide II	Proteins	[5], [6]
	1520 ^	ν(C-C) aromatic Amide II C=N or C=C	Phenolic compounds Proteins Nucleic acids?	[2], [3], [4], [5], [6]
	1254 *	δ(OH) Amide III $\nu_a(CH_2)$	Cutin / polysaccharides Proteins Phospholipids	[2], [3], [5], [6]
	1215 ^	$\nu_a PO_2$ Amide III	Phosphate Proteins	[5], [6]
Early Infection	**1747**	ν(C=O) ν(C=C) Aliphatics	Polysaccharides Lipids (fatty acids) Suberin	[2], [4], [5], [6]
	1701 *	ν(C=O· · ·H)	Fatty acid esters Cutin	[3], [5], [6]
	1659 +	ν(C=C) Amide I	Pectin Proteins	[4], [5], [6]
	1632 *	ν(C=C) ν_s(C-C) ring Amide I	Phenolic compounds Proteins	[2], [3], [4], [5], [6]

	1366	$\delta(CH_2)$ $\nu_s(COO)$ $\delta_s(CH_3)$	Cutin and waxes Cellulose Fatty acids Sesquiterpenes	[2], [4], [5], [6]
	1246 *	$\delta(OH)$ Amide III $\nu_a(CH_2)$	Cutin / polysaccharides Proteins Phospholipids	[2], [3], [5], [6]
Late Infection	**1724**	$\nu(C=O)$	Cutin and lipids Pectin / polysaccharide Phenolic ester	[1], [2], [3], [4], [6]
	1651 +	$\nu(C=C)$ Amide I	Pectin Proteins	[1], [5], [6]
	1582 ^	Amide II	Proteins	[5], [6]
	1520 ^	$\nu(C-C)$ aromatic Amide II C=N or C=C	Phenolic compounds Proteins Nucleic acids	[1], [2], [3], [5], [6]
	1466	$\delta(CH_2)$	Cutin and waxes	[1], [3], [4], [6]
	1215 ^	ν_aPO_2 Amide III	Phosphate Proteins	[5], [6]

Overlap between wavenumbers ±10 was considered; * indicates overlapping wavenumbers between damaged and early infection; ^ between damaged and late infection; + between early and late infection. **Bold** wavenumbers indicate class specific wavenumbers. Table References: [1] Butler et al. 2015; [2] Butler et al. 2017; [3] Heredia-Guerrero et al. 2014; [4] Largo Gosens et al. 2014; [5] Movasaghi et al. 2008; [6] Ord et al. 2016.

Spectral alterations in plant surface structures of tomato, related to plant-pathogen interactions have been previously identified and may be related to conserved changes in epidermal surface structures in response to stress through the reactive oxygen species (ROS) network. Plant-pathogen interactions induce complex signaling networks leading to the induction of the hypersensitive response (HR) and/or systemic acquired resistance (SAR), both of which involve significant alterations to metabolism including lignification, suberization, callose deposition, changes in ion fluxes and lipid peroxidation (Camejo et al. 2016). The HR also involves the activation of programmed cell death (PCD). This is often accompanied by an oxidative burst generating ROS in the form of superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH) accumulation (Apel and Hirt 2004; Hakmaoui et al. 2012; Suzuki et al. 2011). More generally, ROS signatures are altered in response to abiotic and biotic stresses alone and in combination (Camejo et al. 2016; Choudhury et al. 2017). Further, the oxidative burst, initiated during plant-pathogen interactions with fungi, generates ROS, which influence structural features of both cuticle and cell wall (AbuQamar et al. 2017). Part of the response to damage and pathogen attack, specifically at the late ripening stage is accelerated fruit softening caused by cutin depolymerization, which occurs naturally during the ripening program (Saladie et al. 2007). Observed changes are therefore likely associated with a stress response initiated by fungal infection at a distance (in this case infection at the stem scar) and not caused by fungal released cutinases leading to cutin hydrolysis and depolymerization (Chen et al. 2001). The region 1750-1700 cm^{-1} has been implemented in the measurement of cutin in tomato cuticles, with the potential to determine the degree of cutin esterification (España et al. 2014). This region was not only identified as a major cuticle component of intact tomato fruit (Table 1) but was also extracted by PCA-LDA for all classes (Table 2) making this spectral region a potentially robust biomarker indicative of spectral alterations associated with cuticle dependent shelf-life and pathogen susceptibility. ROS signatures, or more specifically downstream targets of ROS present in epidermal surface structures such as the cuticle and cell wall, may therefore offer suitable targets for the detection and potential quantification of both abiotic/biotic stresses in various combinations (AbuQamar et al. 2017; Choudhury et al. 2017). Wavenumbers associated with the categories of damage, early, and late infection (Table 2), have also been identified as biomarkers related to abiotic and biotic stress in the epidermal surface structures of intact leaves of *Acer pseudoplatanus* (Sycamore) (Ord et al. 2016). Importantly, in this and the study by Ord et al. (2016), ATR-FTIR

spectroscopy coupled with the composite technique PCA-LDA was employed emphasizing the effectiveness of this technique to extract biochemical information from dynamic biological processes. Spectral biomarkers identified in *A. pseudoplatanus* were associated with abiotic stresses caused by ozone and vehicle air pollution, as well as biotic stress caused by the tar spot leaf fungus *Rhytisma acerinum*. Changes in the cuticle and cell wall, as well as ROS signaling, are early events in the response of plants to environmental stress making it plausible that certain biochemical and biophysical changes occurring in plant surface structures in response to stress are conserved between species. Consequently, the observed alterations in the spectral signature of *A. pseudoplatanus* leaves (Ord et al. 2016) may be linked to the generation of ROS in response to stress providing the connection between these biomarkers. This would explain the appearance of spectral biomarkers in tomato fruit related to damage and biotic stress, which have been previously associated with both abiotic and biotic stresses in surface structures of the distantly related *A. pseudoplatanus*. Although spectral biomarkers identified here in tomato match with stress biomarkers reported previously, the biomarkers occur in different combinations, which may be due to a combination of factors including inter-species differences, difference in tissue type, or differences conferred by disease (stress) specificity. Nevertheless, the identification of such a large number of spectral biomarkers point to strong commonalities between these two, different species, and suggests that spectral alterations relate to dynamic physiological changes pertaining to biotic and abiotic stress responses. While difficult to confirm through spectrochemical analysis alone, once additional data become available, the link between changes in the MIR signature, changes in epidermal structures, plant stress, and specific signaling pathways such as ROS, will become increasingly clear.

4.3.3 *In Vivo* Spectral Characterization of Sour Rot Pathogen *Geotrichum candidum*

Interaction of *G. candidum* with tomato fruit *in vivo* appears to alter the MIR spectrum characteristic of typical fungi. The fungus *G. candidum* was measured on the tomato fruit as depicted in Figure 4.1f at 96 h post puncture. To date, MIR has been primarily used to study fungal pathogens from isolated and prepared samples (Salman et al., 2012, 2010). *G. candidum* is an economically important pathogen as it induces sour rot in many fruit and vegetable crops including tomato (Cantu et al. 2008). Its ubiquitous occurrence, as part of the human micro-biome, soil, as well as horticultural processing equipment, makes pathogenic strains of *G. candidum* a threat to

crops (Thornton et al. 2010). Further, *G. candidum* can improve the conditions for infection by other pathogens thus contributing to further infection or synergistic pathogen interactions (Suzuki et al. 2014; Wade et al. 2003).

Figure 4.4 shows the ATR-FTIR fingerprint spectrum of *G. candidum in vivo* on tomato fruit. The main six vibrational bands of *G. candidum in vivo* are shown in Table 3. Identified vibrational bands are distinct from those of tomato fruit (Figure 4.2 and Table 4.1) and contain several absorbance peaks consistent with those of other fungal pathogens (Salman et al., 2012, 2010). Absorbance peaks at 1639, 1547, 1404, and 1034 cm^{-1} could be assigned to a typical fungal MIR spectrum (Salman et al., 2012, 2010). In comparison, vibrational bands at 1342 and 1238, which are prominent peaks of *G. candidum in vivo,* appear to be much less pronounced or even absent depending on the fungal species under study (Salman et al. 2012). Main absorbance peaks of *G. candidum in vivo,* show vibrational modes associated with proteins between 1639-1342 cm^{-1}. Specific absorbance peaks over this region include 1639, 1547, and 1404 cm^{-1}, corresponding to the fundamental protein vibrations amide I, amide II, and (C-N) vibration respectively (Movasaghi et al. 2008; Salman et al., 2012, 2010). Vibration at 1034 cm^{-1} is also readily identified as belonging to the Chitin (C-O) bond (Salman et al., 2012, 2010*).* Absorbance at 1639, 1547, 1404, and 1034 cm^{-1} are thus all consistent with those previously characterized in fungal isolates of *Colletotrichum, Fusarium, Rhizoctonia* and *Verticillium* species (Salman et al., 2012, 2010). However, the vibrational mode identified here at 1238 and 1342 cm^{-1} does not appear to be a common constituent of other fungal pathogen isolates (Figure 4.4). Phosphate (PO_4^{2-}) vibrational band at 1238 cm^{-1} is strongly associated with nucleic acids such as part of the DNA or RNA phosphate backbone (Mogashavi et al. 2008). Polysaccharide vibration (CH_2), atypical of fungi was also identified as a strong peak of *G. candidum* as part of the tomato fruit-pathogen complex (Figure 4.4). It is likely that the discrepancy between the spectrum of *G. candidum* and those of other species is a result of *in vivo* analysis. The unique interaction between fungi and their host plants could influence the measured composition of the fungus, when compared to MIR spectra of fungal isolates, which are homogenized and taken out of their biological context. Although it cannot be ruled out that lack of sample preparation (dehydration and homogenization) prior to spectral acquisition, led to a higher water content and more heterogeneous arrangement, which influenced the MIR spectrum (Figure 4.4). The fundamentally different biochemical composition of fungal pathogens to that of tomato fruit is reflected in their respective MIR fingerprint spectra (Figures 4.2 and 4.4). This

fundamental difference in composition has led to the direct detection of fungal pathogens within plant tissues using differences in MIR spectral data. However, here we demonstrate that the typical fungal spectrum may have very unique features when measured intact. What remain to be seen is whether the differences arise due to simple fungal heterogeneity, or whether the interaction between plant and pathogen is the driving force for changes in its MIR fingerprint. Regardless, the characterization of pathogens in their native state, and as part of *in vivo* host-pathogen systems, is necessary to fully evaluate MIR for the non-destructive and rapid analysis of plant-pathogen interactions outside of the laboratory under the many variable conditions in which they occur.

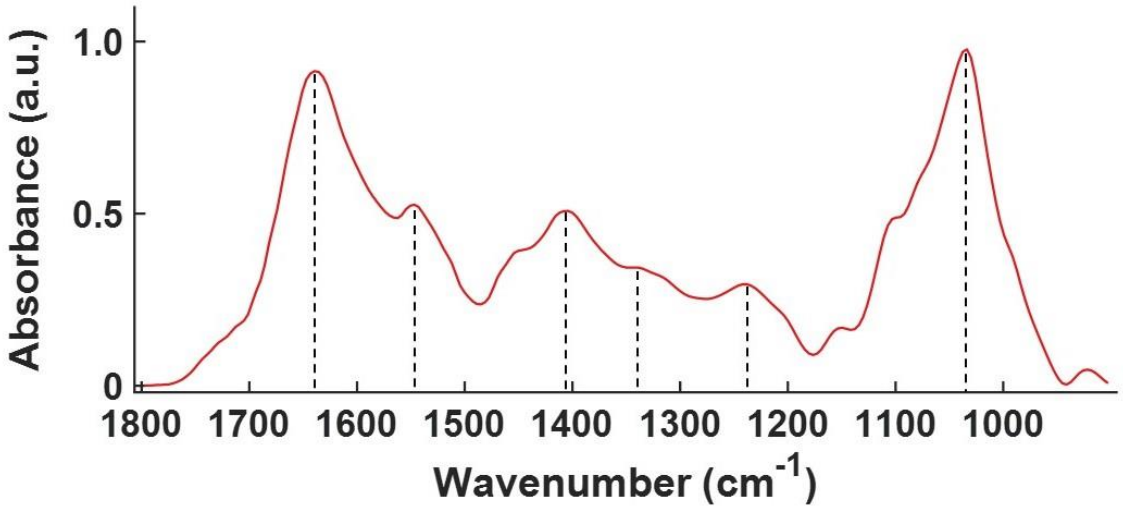

Figure 4.4 ATR-FTIR fingerprint spectrum of *in vivo* sour rot pathogen *G. candidum* present on tomato fruit at the 96 h late infection stage.

Table 4.3 Primary absorbance peaks of fungal pathogen *G. candidum* in vivo on tomato fruit

Wavenumber (cm^{-1})	**Vibrational Mode**	**Biochemical Assignment**	**References**
1639	Amide I	Proteins	[1], [2]
1547	Amide II	Proteins	[1]
1404	ν(C-N)	Proteins	[2]
1342	ν(CH_2)	Polysaccharides	[3]
1238	ν(PO_4^{2-})	Nucleic Acids	[3]
1034	ν(C-O)	Chitin	[1]

[1] Salman et al. 2012; [2] Salman et al. 2010; [3] Movasaghi et al. 2008

4.3.4 Autonomous Indirect Detection of Damage and Infection Based on Alterations to Tomato Fruit Surfaces

Diagnostic classifiers based on PCA-LDC are effective at detecting tomato fruit damage and infection indirectly and autonomously. PCA-LDC is one of many classifier algorithms used as training/test datasets to evaluate the potential for autonomous classification based on MIR spectra (Butler et al. 2017; Strong et al. 2017). To evaluate the potential for autonomous detection of damage, early, and late infection, compared to healthy shelf-life matched controls using MIR, spectra of intact tomato fruit were used as training/test datasets for the PCA-LDC classifier (Figure 4.5). Discrimination of classes using PCA-LDC has recently been applied to plant tissues with high accuracy (Butler et al. 2017). Classification of healthy controls, compared to their initially damaged but non-infected counterparts, showed the lowest observed accuracy at 78% for healthy controls, while freshly damaged tomatoes were identified correctly 83% of the time (Figure 4.5a). In comparison, tomato fruit showing early signs of sour rot were accurately classified at 97%, and 92% for healthy controls at 48 h post puncture (Figure 4.5b). Late-stage *G. candidum infect*ed tomatoes correctly classified 83% similar to freshly damaged tomatoes, was in contrast to the classification of control group at 96 h, which showed a classification accuracy of 96% (Figure 4.5c). This was consistent with the separation observed along the primary LD for these classes (Figure 4.3), as well as with the classification rates achieved by Butler et al. (2017) investigating calcium nutrient deficiency in tissues of *Commelina communis*. Interesting is the slightly higher classification accuracy at early compared to late stage infection (Figure 4.5b and 4.5c). Late stage infection leading to tissue breakdown and fruit softening is likely more closely related to the natural ripening process represented by control fruit at 96 h across late infection leading to slightly lower classification accuracy (Figure 4.5c). Alternatively, the switch from damage to pathogen response may be more pronounced in comparison to shelf life changes at 48 h, which may lead to the higher classification compared to the 96 h analysis point (Figure 4.5b). This is beneficial as detection of early infection is favorable over detection at later disease stages.

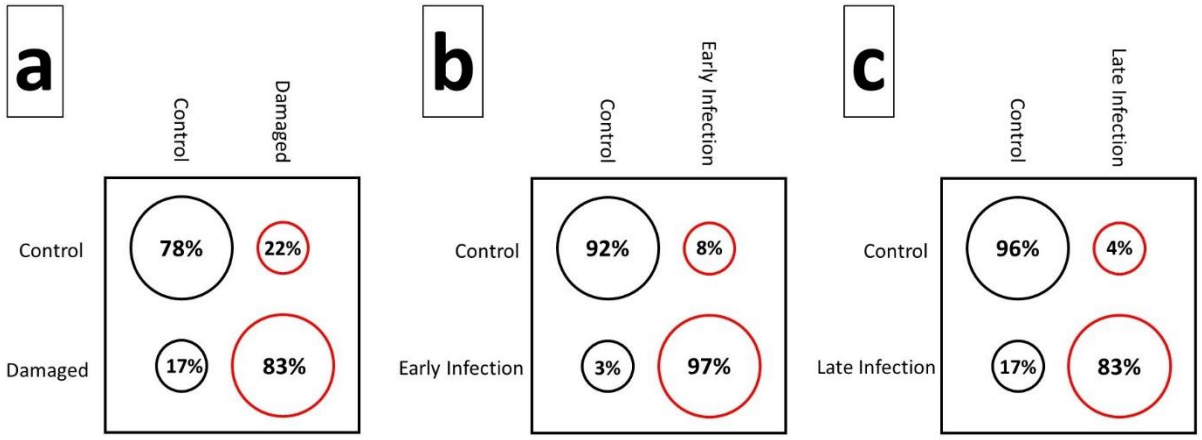

Figure 4.5 Classification rates (%) of damage, early, and late infection compared with shelf-life matched controls, extracted from PCA input to linear discriminant classifier (PCA-LDC).

These collective data suggest that correct classification of infected tomato fruit improves with disease progression. Demonstrating that this is possible indirectly based only on changes in fruit epidermis not yet afflicted by pathogens, will be important to be able to detect damaged tomato fruit, prior to the development of the symptoms of fungal infection in order to reduce food waste through the repurposing of the affected crops. In addition, the early detection of fungal infection would help prevent the effects and spread of post-harvest disease. It is well documented that damage to delicate fruits and vegetables leads to rapid spoilage (Tournas 2005) and thus early symptoms of damage may also serve as a pre-symptomatic indicator for imminent infection by ambient microorganisms. To this end, classifier performance may be further optimized by increasing the number of factors (PCs) fed into the LDC. For commercial development, appropriate training and test datasets would likely improve classification accuracy further. Nevertheless, preliminary classification accuracy of around 80% upwards is promising and certainly provides precedence for further development of spectrochemical analyses as a tool for crop protection.

4.4 Conclusions and Future Perspectives

Spectrochemical analysis combined with multivariate analysis offers a non-destructive sensor technology for the analysis of intact crops, active pathogens, and plant-pathogen interactions. Spectral characterization of intact tomato fruit showed prominent components from the cuticular layer of the plant epidermis including cutin, phenolic compounds, waxes, and VOCs.

During healthy growth and plant-environment interactions, these compounds are notably modified with consequences for fruit quality and thereby provide unique groups of compounds serving as targets of dynamic processes pertaining to crop biology. At the environmental interface, the cuticle is of specific importance due to its role, as part of the cell wall, in the determination of fruit qualities such as susceptibility to cracking and pathogen infection (Isaacson et al. 2009; Lara et al. 2014).

Multivariate analysis (PCA-LDA) can effectively discriminate healthy and compromised tomato fruit, 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, 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. Disease specificity based on unique spectral markers is tentatively linked to complex and evolving stress responses. While the exact connection between spectral biomarkers of 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.

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

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 analyze 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 in the near future.

4.5 Supplementary Materials

SUPPORTING INFORMATION

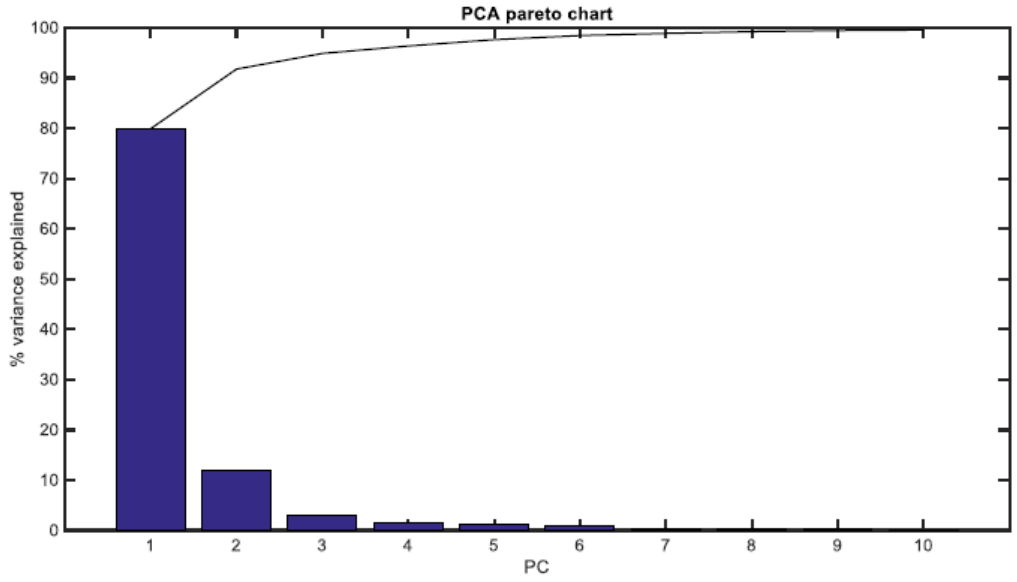

Figure S1: PCA optimization using the Matlab *pareto*-function.

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Chapter 5: Attenuated Total Reflection Fourier-Transform Infrared Spectroscopy Coupled with Chemometrics Directly Detects Pre- and Post-Symptomatic Changes in Tomato Plants Infected with *Botrytis cinerea*

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Abstract

Sensor-based detection of pests and pathogens in a high throughput and non-destructive manner is essential for mitigating crop loss. Infrared (IR) sensors in the form of vibrational spectroscopy provide both biochemical information about disease, as well as a large number of variables for chemometrics. This approach is highly adaptable to most biological systems including interactions between plants and their environments. Fast-acting necrotrophic fungal pathogens present a specific group of pests with adverse effects on food production and supply and are therefore pertinent to food security. *Botrytis cinerea* and *Solanum lycopersicum* are models for the study of fungal and crop biology respectively. Herein we use a compact mid-IR spectrometer with attenuated total reflection (ATR) attachment to measure the plant-microbe interaction between *S. lycopersicum* and *B. cinerea* on intact leaves at the whole-plant level. Chemometric models including exploratory principal component analysis (PCA) solely, and as a classifier in combination with linear discriminant analysis (PCA-LDA) are applied. Fingerprint spectra (1800-900 cm^{-1}) were excellent discriminators of plant disease in both visually symptomatic as well pre-symptomatic plants. Major biochemical alterations in leaf tissue as a result of infection are discussed. Diagnostic potential for automatic decision-making platforms is shown by high accuracy rates of 100% for detecting plant disease at various stages of progression.

Keywords: Infrared spectroscopy, Tomato, *Botrytis cinerea*, Chemometrics, Crop biology, Pest Detection, Sensors

5.1 Introduction

The challenges to sustainable crop production and supply are considerable and include population rise, depletion of natural resources, and climate change. Combined with these, crop loss and food waste to either pests and pathogens, or due to general waste, puts a heavily burden on humans and the environment (Wunderlich and Martinez 2018). Conventional but outdated farming strategies are compounding these issues because traditional methods are only being slowly replaced by more sustainable approaches. The example of fungicides use illustrates that these are only marginally effective, expensive, and yet routinely overused (Bourguet and Guillemaud 2016; Vasileiadis 2017). Alternatives to chemical pest control are thus favorable, especially with the growing concern about harmful residues of crop protection products in food (Singbo et al. 2015). One approach therefore may be to focus on the early detection of plant disease to remediate imminent threats, or prevent further disease spread.

Crop loss to destructive pests and pathogens poses a substantial threat to food security and the economy. Around one third of global annual crop loss can be attributed to pests and pathogens (Oerke 2006) including macroscopic pests such as weeds, herbivorous animals, and insects, and microscopic pathogens such as viruses, bacteria, and fungi. In contrast to macroscopic pests, microscopic pathogens are invisible without the use of tools, becoming apparent in horticultural environments only through adverse effects (symptoms) they cause. Moreover, the current horticultural standard for detection of these pathogens relies on the appearance of plant disease symptoms and is thus subjective (Mahlein 2016). Microscopic pests thus remain especially challenging for plant and crop scientists to manage (Williamson et al. 2007), primarily due to their microscopic nature, but also because they employ a vast combination of pathogenic strategies for survival (El Oirdi et al. 2011). The ubiquitous distribution of microbial pathogens and their long-standing interactions with host plants throughout plant evolution makes these plant-pathogen interactions highly complex and diversified (Dayan et al. 2009).

Fungi are pests that have evolved to invade plants by various means and with varying consequences. Three main strategies distinguish fungal pathogens. These include biotrophism, hemi-biotrophism, and necrotrophism. Biotrophs complete their life cycle without killing the host plant and rather establish a nutritional mode that depends on living plant cells (Vleeshouwers and Oliver 2014). Hemibiotrophs begin their life cycle with a biotrophic strategy and can switch to a necrotrophic mechanism, which results in cell death (Vleeshouwers and Oliver 2014).

Necrotrophic pathogens therefore have the potential to destroy entire plants and crops rapidly. *Botrytis cinerea* is an important necrotroph capable of causing extensive damage to food crops, both pre- and post-harvest, and is therefore widely studied as a model necrotoph. High genetic diversity, various modes of attack, a broad host range, and the ability to remain quiescent, make *B. cinerea* a formidable threat to crops infecting most plant tissues including seeds, seedlings, roots, stems, leaves, flowers, young and mature plants, fruit, and plant waste (Williamson et al. 2007). The range of host species numbering over 200 provide a diverse source of potential inoculum which can be ubiquitous in horticultural environments as mycelia, conidia, or prolonged periods as sclerotia, making control of this pathogen particularly difficult (Elad and Stewart 2007). Many fungicides have become ineffective over time due to pathogen adaptation leading to potential overuse of these pesticides making this approach costly and unsustainable in a horticultural context (Lechenet et al. 2014, 2017). Therefore, new approaches for the management of pathogenic fungi are required to optimize crop protection measures. To this end, a more detailed understanding of plant-pathogen interactions and the way they manifest during crop cultivation is an essential prerequisite for improving crop utilization and minimizing crop loss due to microscopic pathogens.

The rapid and early detection of fungal pathogens in the crop-growing environment remains a key challenge. This is especially so when there is requirement for the non-destructive analysis of crops during growth leaving them unaltered in the process, for example, when monitoring whole plants and crops in the field, or produce within the food supply chain. These criteria favor adaptable sensors capable of analysis at both pre- and post-harvest stages without damaging the crop; this is in marked contrast to destructive methods which require reagent preparation, labeling, extensive sample preparation, and provide potentially limited information with regards to pathogen detection. Within the array of non-destructive sensors, spectroscopic methods have been used successfully in horticultural settings and are becoming increasingly adaptable for field applications (Egging et al. 2018; Farber and Kurouski 2018; Trebolazabala et al., 2013, 2017; Yeturu et al. 2016). These methods have the advantage of being capable of the fast, non-destructive analysis of a diverse set of sample types, which provide the biochemical information on which to build models of disease specificity (Martin et al. 2010). Sensor-based early disease detection has the potential to contribute to the sustainable intensification of crop production, by improving the effectiveness with which crop protection measures are applied and

thereby considerably reducing disease spread through precision agriculture.

Progress in sensor technology, computational analysis, and established machine learning methods offers new opportunities for this approach through the development of mobile devices for the early detection and identification of plant disease (Behmann et al. 2014). Surface techniques have been widely used for analytical chemistry and have recently been developed as sensors for heterogeneous biological materials including cells, tissues, and whole plants. Analytical surface techniques include infrared vibrational spectroscopy, a non-destructive sensor conferring data with high chemical specificity in the form of a highly characteristic infrared spectrum. The ability to measure almost any sample type without preparation or labeling using mid-infrared (MIR) vibrational spectroscopy in biology (biospectroscopy), has made it a valuable tool for studying biological systems at the tissue, cell, and molecular levels in plants and crops (Skolik et al. 2018b). One method that has stood out in the biological context and adaptability to *in vivo* measurements non-destructively is attenuated total reflectance (ATR) Fourier transform infrared (FTIR) spectroscopy, which uses a high refractive index material interface to make direct sample contact, resulting in highly reproducible spectra from living tissues (Chan and Kazarian 2016). Further benefits of ATR-FTIR is the very well-defined depth of interrogation into the sample and its ability to circumvent the effects of water in plant leaves when practically applied (Butler et al. 2015). This makes MIR spectroscopy in ATR mode favorable for whole tissue analysis including whole plants.

Extracting information from complex biological samples requires computational analysis such as linear and non-linear data models (chemometrics) (Morais and Lima 2017). Chemometrics such as multivariate analysis using supervised and unsupervised methods including principal component and linear discriminant analysis (PCA and LDA respectively) as well more complex models including support vector machine (SVM) and artificial neural networks (ANN) have also been used in various amalgamations to answer questions in the biomedical, environmental, plant and crop sciences (Trevisan et al. 2012). Biospectroscopy in combination with advanced computational analysis therefore has the potential to contribute to the development of sustainable farming practices and precision agriculture, and to help achieve the goal of producing more food with the same amount of land by reducing crop losses to pests and pathogens through the adoption of ‘high-tech farming’ approaches.

Tomato *S. lycopersicum* is an ancient crop which is widely used for the study of crop

biology (Kimura and Sinha 2008). Plants and fruit of tomato are among the many species susceptible to pathogen infection by *B. cinerea* at all stages of development (Elad and Stewart 2007; Williamson et al. 2007). Tomato interaction with fungi thereby presents an excellent model to investigate challenging plant-pathogen interactions with rapid disease progression for the purposes of sensor development. This study applies MIR biospectroscopy within a realistic growing environment to investigate the effectiveness of this approach within an agricultural context using the interaction between glasshouse-grown tomato (*S. lycopersicum* cv. Moneymaker) and *B. cinerea*. Chemometric approaches including PCA and PCA-LDA were used to investigate spectral alterations in leaves in response to *B. cinerea* at the whole-plant level. PCA is an unsupervised multivariate analysis approach that investigates dataset variance without considering class label and thus provides insight into dataset variance, specifically whether the biological effects under investigation are the main source of variance, or whether supervised models (LDA) are required to extract class-specific differences which are subtler than can be extracted via PCA alone. Further, both PCA and LDA are linear multivariate models that provide loadings, which may be interpreted as spectral biomarkers. This provides a novel approach, combining exploratory multivariate analysis (PCA and LDA) for biomarker extraction, with diagnostic evaluation through classifier simulation. Evaluated in the whole-plant context investigating primary changes in leaf tissue due to infection and disease progression, as well as classification of infection category based on class labels (time after infection) to show the practical applicability of portable ATR-FTIR spectroscopy for the study of plant-pathogen interactions.

5.2 Materials and Methods

5.2.1 Plant Growth Conditions

Tomato plants, *Solanum lycopersicum* cv. Moneymaker (Thompson and Morgan, Ipswich, UK) were grown from seed in 1 L pots containing Levington's M3 growth medium (Levington Horticulture Ltd, Ipswich, UK) for 3 weeks under glasshouse conditions (40-60% relative humidity, 23.5-28.5°C) with an 18/6 h day/night cycle (minimum illumination 500 µmol m^{-2} s^{-1} at the plant canopy from 600 W metal-halide lamps). Plants were watered daily between 8-9 am to holding capacity.

5.2.2 *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 Pollastro 1991), were placed in the center 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: Fluora L 18 W/77, Osram, Munich, Germany) for 7 days to induce sporulation (Schumacher 2017). Loose spores were washed into a 50 mL falcon tube, 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 15,000 rcf at 15°C. Supernatant was removed by decanting and spores were re-suspended and combined in 15 mL molecular grade water (Sigma Aldrich, St. Louis, Missouri). The concentration of spores determined via hemocytometer was adjusted to 5×10^5 spores mL^{-1} in 0.05 M KH_2PO_4 and 33 mM glucose respectively (Asselbergh et al. 2007). This solution was prepared 3 h 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 ml 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, Ulm, Germany) producing a 50 µm droplet diameter 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 were assessed visually at the three measured time points and used as class criteria. Detailed description of symptoms is summarized in Table 5.S1 and shown in Figure 5.1. Described symptoms and time of onset after exposure to fungal pathogen were consistent with various stages of infection as previously described (Asselbergh et al. 2007; Audenaert et al. 2002; El Oirdi et al. 2011) and thus covered the range of disease progression relevant for crop protection.

5.2.3 ATR-FTIR Spectroscopy

Vibrational spectra from tomato plant leaves were taken using a Bruker Alpha IR spectrometer with Platinum ATR attachment (Bruker Optics, UK). Spectra were acquired over the range 4000-400 cm^{-1} with a spectral resolution of 8 cm^{-1}, 32 co-additions and a mirror velocity of 7.5 kHz for optimum signal to noise ratio and acquisition speed. Background spectra were taken prior to each sample to account for the ambient atmosphere. The diamond ATR crystal defined a spatial resolution (sampling area) of 1 mm^2. ATR cleaning wipes containing isopropyl-alcohol (Bruker, UK) were used to clean the ATR diamond crystal between sample measurements.

A total of 16 leaves (4 controls, 4 infected plants) were measured for the three different categories (pre-symptomatic, intermediate symptomatic, advanced symptomatic). Ten spectra were collected per each leaf, resulting in 80 spectra for each category (240 spectra in total). Five spectra were taken from each of the two main leaflets, comprising each biological replicate (whole plant). Four plants (n=4) were measured for controls and infected plants respectively at each timepoint. Spectra taken from symptomatic leaves at 96 and 144 h post infection were from regions of remaining healthy tissue (for an example of acquisition points on healthy and infected leaves, see Figure 5.1). Plants were maintained under a portable 600 W high-pressure sodium lamp (Omega lighting, Berkeley, CA) source (minimum of 500 µmol m^{-2} s^{-1}) during spectral acquisition to simulate glasshouse conditions during whole plant analysis.

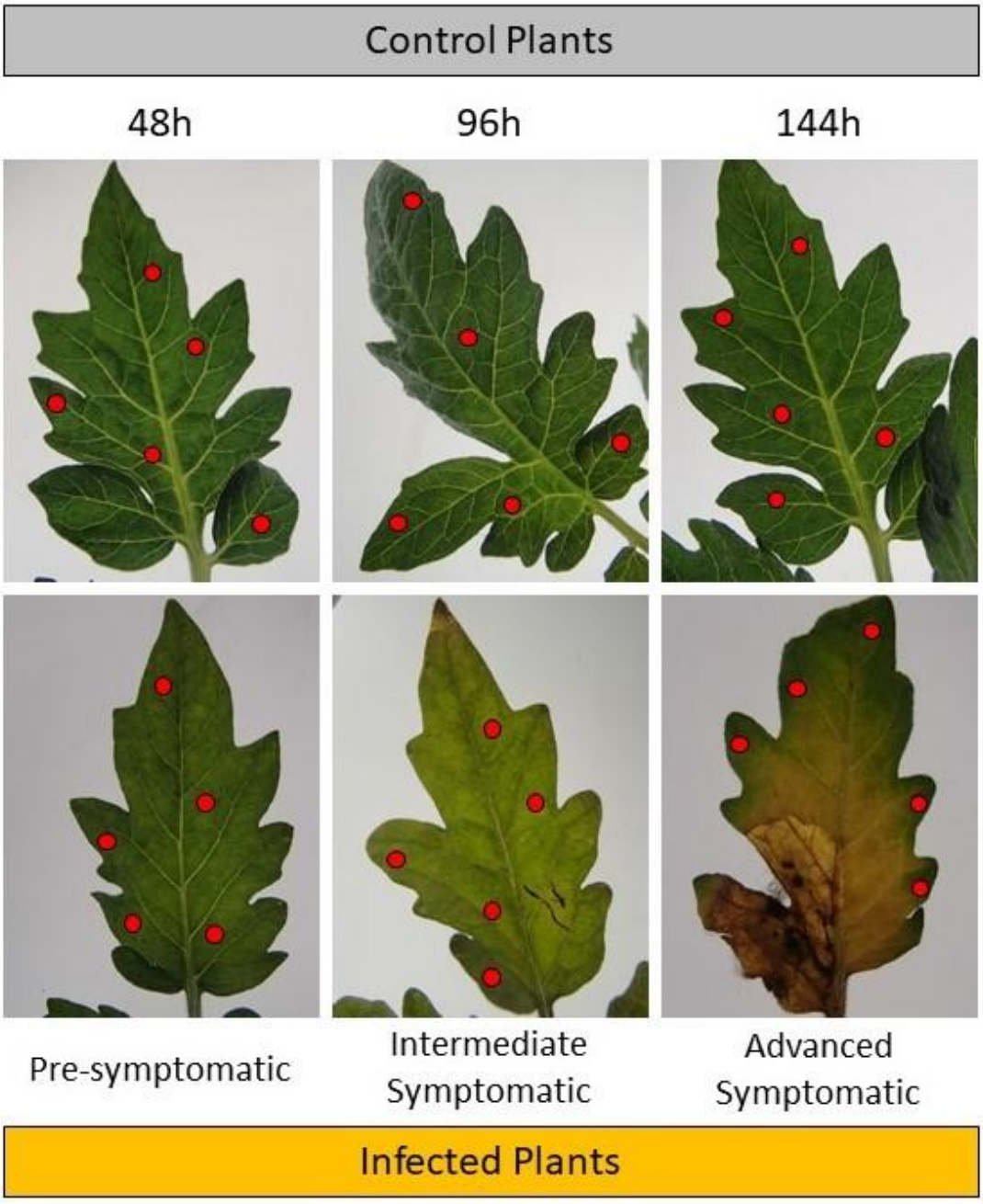

Figure 5.1 Examples of spectral acquisition points on leaves of whole tomato plants. Measurements were taken from healthy looking tissue of infected leaves as shown (this was only necessary in symptomatic tissue (infected leaves at 96 and 144 h).

5.2.4 Computational Analysis

All pre-processing and data analysis were carried out using the PLS toolbox version 7.9 (Eigenvector Research, Inc., WA, USA) in conjunction with MATLAB 2016a (The Math Works, MA, USA). Pre-processing of raw spectra was performed by selecting the MIR fingerprint region (1800–900 cm^{-1}), baseline correction using automatic weighted least squares (AWLS) followed by vector normalization. Initially, the mean-centered pre-processed spectral data were evaluated by means of principal component analysis (PCA) (Bro and Smilde 2014). PCA is an exploratory analysis method that reduces the original variables (i.e., wavenumbers) to a few number of principal components (PCs) accounting to the majority of the original data variance. Each PC is orthogonal to each other, where the first PC accounts to the maximum explained variance followed by the second PC and so on. The PCs are composed of scores and loadings, where the first represents the variance on sample direction, thus being used to assess similarities/dissimilarities among the samples; and the latter represents the contribution of each variable for the model decomposition, thus being used to find important spectral markers. PCA was the method of choice for analyzing this dataset since it is simple, fast, and combine exploratory analysis, data reduction, and feature extraction into one single method. PCA scores were used to explore overall dataset variance and any clustering related to plant infection independent of class label, while the loadings on the first two PCs were used to derive specific biomarkers indicative of plant infection at different stages (infection category). PCA was used to explore overall dataset variance, which was related to plant infection independent of class label. This was to determine whether the dataset variance was caused primarily by the infection (observed symptoms) and to rule out other underlying effects such as naturally occurring tissue heterogeneity.

Discriminant analysis was performed to distinguish the samples into control and infected in a predictive multivariate fashion. This was achieved by means of principal component analysis linear discriminant analysis (PCA-LDA). In PCA-LDA, a linear discriminant analysis (LDA) classifier is employed in the PCA scores (Morais et al. 2018). LDA finds the best linear discriminant direction between the groups, maximizing the distance between the classes and minimizing the samples distance within each class. The PCA-LDA classification score for each sample ($cf(\boldsymbol{t}_i)$) can be estimated in a non-Bayesian form by a Mahalanobis distance calculation as follows (Morais et al. 2018):

$$cf(\boldsymbol{t}_i) = (\mathbf{t}_i - \bar{\mathbf{t}}_k)^{\mathrm{T}} \mathbf{C}_{\mathrm{pooled}}^{-1} (\mathbf{t}_i - \bar{\mathbf{t}}_k) \quad (1)$$

where $\mathbf{t}_i$ is a vector containing the PCA scores for all selected PCs for a given sample *i*; $\bar{\mathbf{t}}_k$ is the mean PCA scores vector for class *k*; $\mathbf{C}_{\mathrm{pooled}}$ is the pooled covariance matrix; and the superscript T represents the matrix transpose operation.

PCA-LDA models were built using 70% of the samples in the training set, where the number of selected PCs was optimized *via* 10-k fold cross-validation. The model validation was performed with 30% of the samples, where metrics such as accuracy, sensitivity and specificity were estimated. The sample splitting into training and validation sets was performed using the Kennard-Stone algorithm (Kennard and Stone 1969). For this dataset, four PCs were sufficient to account for more than 95% cumulative variance in each case (Table S5.2).

5.3 Results and Discussion

5.3.1 *Botrytis cinerea* Infection Induces Spectral Alterations in Tomato Plant Leaves

Spectral alterations are observed in leaf tissue of *B. cinerea* infected tomato plants compared to healthy control plants. Primary absorbance peaks of tomato leaves, determined from the class means from pairwise comparisons of control and infected tissue over time (Figure 5.2), show the strongest peaks in the carbohydrate fingerprint region (1200-900 cm^{-1}), as well as the upper fingerprint (UF) region from 1800-1500 cm^{-1}. While the UF region is generally associated with strong protein vibrations, this region also contains vibrations from water, lipids, nucleic acids, as well as various other compounds (Berthomieu and Hienerwadel 2009; Movasaghi et al. 2008). Water absorbs strongly in the region around 1600 cm^{-1}, which is part of the fingerprint region under investigation. It becomes apparent however from the class mean spectra comparisons between control and infected plants that the broad peak including the region 1600 cm^{-1} is variable and not decreasing in infected plants compared to controls, which is inconsistent with water loss caused by cuticle breakdown and tissue degradation due infection. It is therefore likely that this region is indeed a mixture of plant compounds that primarily include biomolecules rather than water. The whole-plant study by Butler et al. (2015) also employed the use of ATR-FTIR to circumvent the effects of water on IR measurements, and analysed whole plant leaves of tomato.

In plants, the region between 1800-1500 cm^{-1} is also strongly influenced by polysaccharide and cutin vibrations abundant in the cutinized cell wall of plant epidermis (Movasaghi et al. 2008; Heredia-Guerrero et al. 2014; Dominguez et al. 2011). While overall absorbance is lower over the region 1500-1200 cm^{-1}, several distinct peaks are nonetheless evident. Figure 5.2A-C shows the unprocessed class mean spectra for healthy control plants, versus plants exposed to *B. cinerea*; pre-processed class means (baseline corrected and normalized) are shown in Figure 5.2D-F for comparison (see Figure 5.S1 original raw spectra). Comparison of healthy and infected tomato leaves shows spectral variations in the pre-symptomatic (PS) stage at 48 h (Figure 5.2A and D), at which point plants do not yet show any visual symptoms associated with *B. cinerea* infection (Figure 5.1, 48 h PS). Clear differences at PS stage are only observed in the region from 1800-1500 cm^{-1} (Figure 5.2D), but not over the carbohydrate region between 1200-900 cm^{-1}, which become clear at the intermediate symptomatic (IS) at 96 h and advanced symptomatic (AS) at 144 h (Figure 5.2E and F). Importantly, plants measured at the PS stage developed visual symptoms at later stages, consistent with those observed at IS and AS, confirming the presence of *B. cinerea* at the PS stage. From class mean spectra, clear alterations in the region between 1500-1200 cm^{-1} are not observed until 144 h (Figure 5.2F). Specifically, differences in the carbohydrate fingerprint region are not observed PS, although slight variation is seen between 1750-1400 cm^{-1} (Figure 5.2D). Spectral divergence similar to that seen at PS between 1750-1400 cm^{-1}, is shifted to between 1650-1300 cm^{-1} at IS and clear alterations to the carbohydrate region between 1200-900 cm^{-1} emerge at IS (compare Figure 5.2D and E). The differences in these regions, indicate important alterations in the underlying biochemical architecture of leaf tissue caused by pathogen infection, which is evident from direct comparison of mean fingerprint spectra of control and infected tomato plants. Increased spectral shifts clearly coincide with the development of visual symptoms on leaves. As infection with *B. cinerea* progresses, characteristic tissue changes occur including the described development of large lesions covering most of the leaf area, black necrotic tissue, visible hyphae and conidiophores indicating pathogen sporulation (Figure 5.1 and Table S5.1).

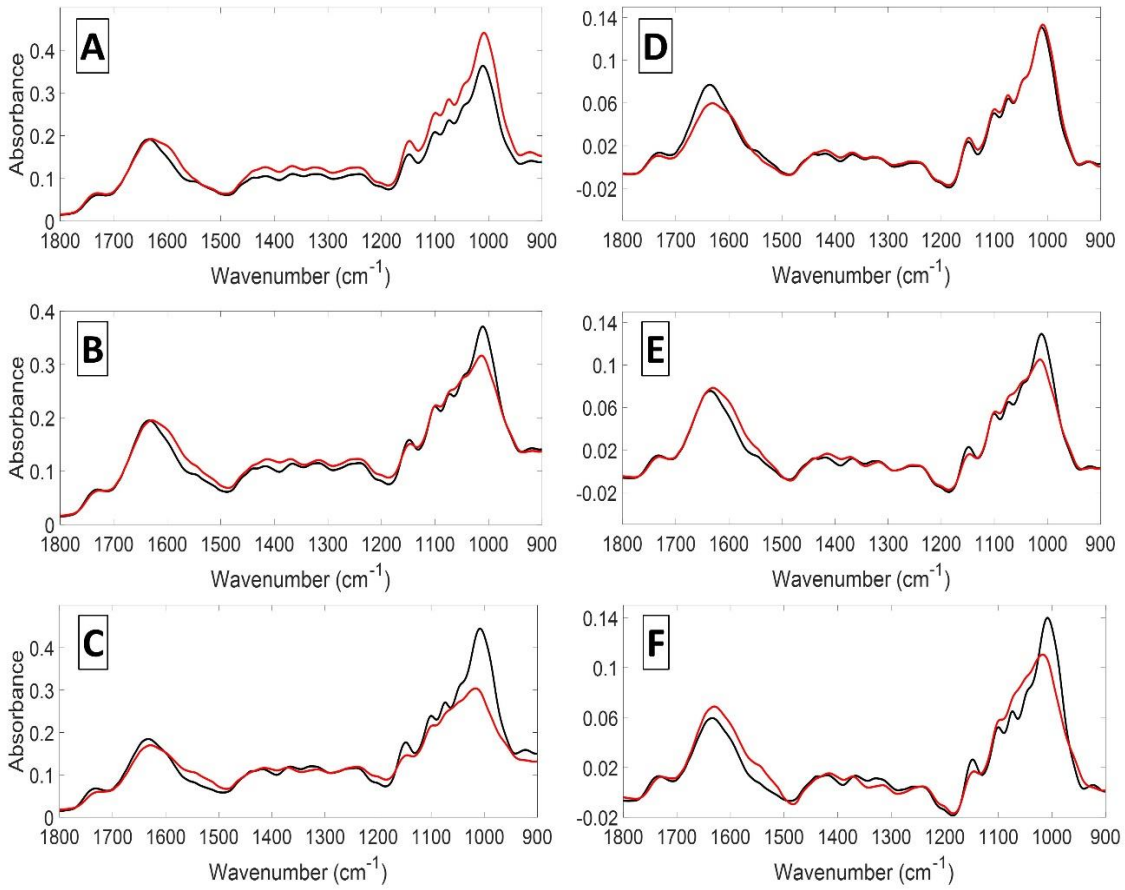

Figure 5.2 Pairwise comparisons of control (black) and *B. cinerea* infected (red) spectra taken from leaves of whole tomato plants over the fingerprint region. Mean spectral classes at 48, 96, and 144 h (A-C) and corresponding pre-processed mean spectra (D-F).

Increasing changes in the carbohydrate and UF regions of plants affected by *B. cinerea* suggest structural changes in plant leaf polysaccharides, proteins, lipids and waxes, as well as potentially nucleic acids. A clear decrease in the absorption over the carbohydrate fingerprint region (1200-900 cm^{-1}) during the infection process, is associated with changes in biochemical composition of polysaccharides present in the surface layers of the epidermis and expected to arise mainly from changes in the cuticle and cell wall (Largo-Gosens et al. 2014; Heredia-Guerrero et al. 2014). Changes in carbohydrates, proteins, and moisture content, are consistent with processes such as cuticle and cell wall degradation resulting in water loss, following pathogen invasion (Asselbergh et al. 2007; Audenaert et al. 2002; El Oirdi et al. 2011). The two main regions, 1750-1500 and 1200-900 cm^{-1} clearly show the largest differences and may be of considerable

importance in the detection of leaf tissue modifications associated with necrotrophic pathogen attack *in vivo* based on MIR spectra, where the UF and carbohydrate sections of the MIR spectrum show the largest variation (Figure 5.2). Although the region between 1500-1200 cm^{-1} showed only slight changes in the class mean spectra over the infection period, it may present an alternative target region to probe the subtler changes occurring *B. cinerea* infection, which can only be extracted through multivariate analysis of the fingerprint spectral data.

5.3.2 Disease Progression Generates Unique Spectral Profiles and Specific Biomarkers at Distinct Stages of *Botrytis cinerea* Infection

B. cinerea infection generates spectral profiles unique to PS, IS, and AS stages of disease progression. Based on the observable differences in the class mean spectra, unsupervised PCA analysis was performed which showed that *B. cinerea* infection was responsible for at least 85% of the variance observed in the spectral data, using the described data analysis approach. For this dataset, four PCs were enough to account for a minimum of 95% total variance for each group (Table S5.2).

Consistent separation of spectral classes (infection categories PS, IS, and AS) was observed along the first two PCs, which accounted for a minimum of 84.89%% of overall dataset variance. At the PS stage, separation along PC1 accounted for 65.52% of dataset variance, while PC2 accounted for 27.96% accounting for a total of 93.48% variance and producing the best class separation using PCA (Figure 5.3A). Spectra from IS and AS plants also showed clear separation from their respective controls along PC1 and PC2 (Figure 5.3B and C). For plants showing IS, class separation along PC1 and PC2 explained 73.97% and 10.92% of dataset variance, respectively, while AS plants showed PC variance at 63.18% (PC1) and 22.56% (PC2), explaining a total variance of 84.89% and 85.72% respectively. Better class separation and accountancy of variance suggests that biochemical changes were most pronounced in PS stages of plant infection, based on results of unsupervised component analysis. For PS detection of plant disease, it is encouraging that the best separation is achieved for the PS category where no visual symptoms are apparent in contrast to leaves clearly infected and colonized by *B. cinerea* at later stages (IS and AS).

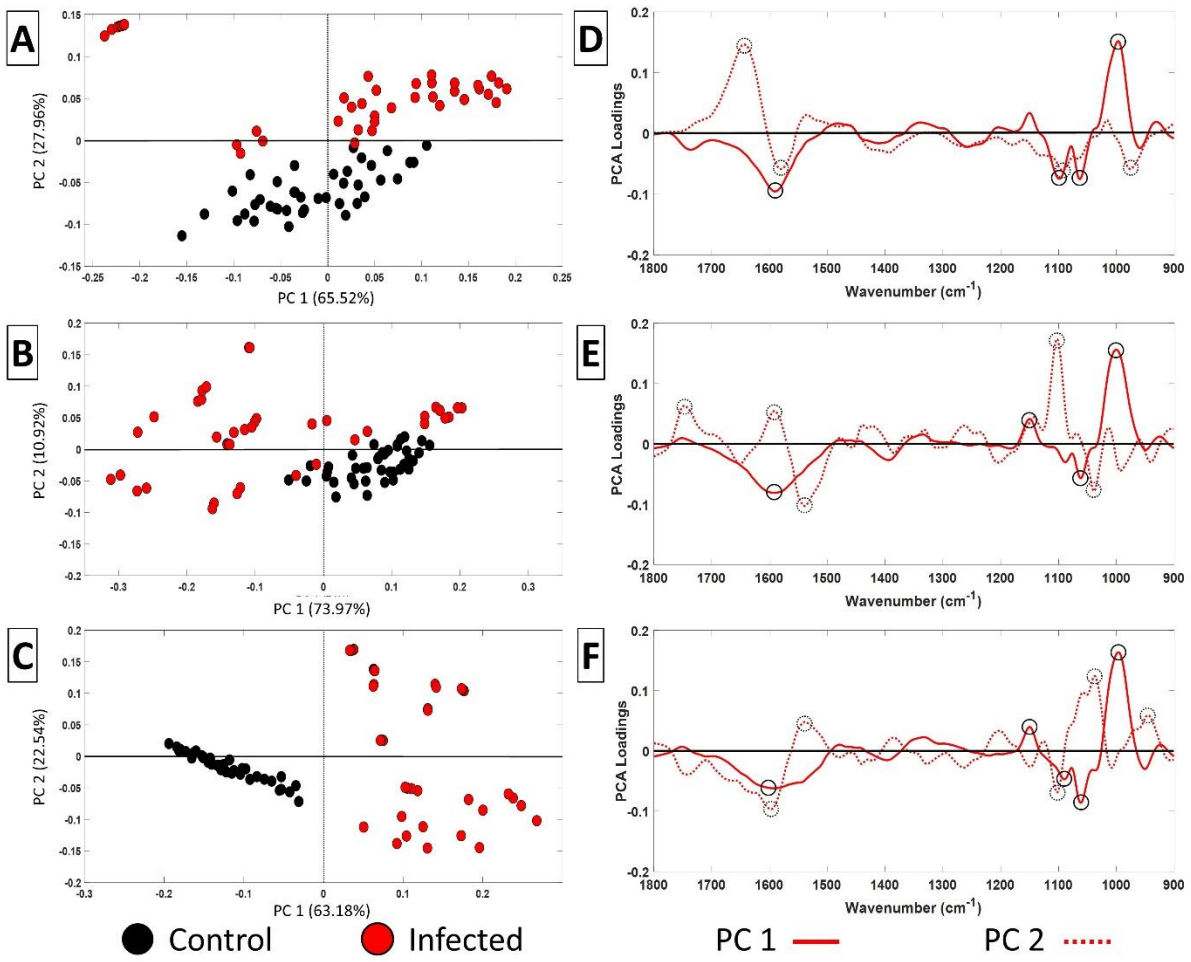

Figure 5.3 PCA 2-dimensional scores plots (A-C) and corresponding loadings (D-F) of pairwise control (black) versus *B. cinerea* infected (red) tomato leaf spectra at 48 (A and D), 96 (B and E), and 144 (C and F) h.

To associate vibrational modes with dataset variance and the observed separation of infected classes based on PC1 and PC2 scores (Figure 5.3A-C), PCA loadings for PC1 and PC2 were generated (Figure 5.3D-F). Major wavenumber peaks within PC loadings represent the main vibrational modes, and thus functional groups and associated compounds, altered because of infection. Compared to PCA scores (Figure 5.3A-C), loadings provide highly specific 'wavenumber biomarkers' responsible for spectral variations between healthy and diseased plants (Kelly et al. 2011). Table 5.1 summarizes these tentatively assigned spectral biomarkers for PS, IS, and AS (Figure 5.3D, E, and F, respectively). Similar wavenumber biomarkers identified over

various regions of the fingerprint spectrum are common to all disease stages, where only a small fraction of wavenumbers, specifically 1748, 1645, 1580, 975, and 945 cm^{-1} are uniquely assigned to a specific category (Table 1; **bold** wavenumbers). Aside from these unique biomarkers similar compounds are identified at all stages of disease progression. Compounds generally related to *B. cinerea* infection are identified over the regions 1600-1590, 1539-1537, 1149-1148, 1105-1088, 1063-1061, 1038-1037, and 1000-995 cm^{-1}, which were consistent with alterations in the two strongest absorbing UF (1800-1500 cm^{-1}) and carbohydrate (1200-900 cm^{-1}) regions identified from the class mean spectra in Figure 5.2. The large degree of overlapping spectral markers between PS, IS, and AS plants indicates alterations in similar biochemical compounds through disease progression over time and thereby links biomarkers from each distinct disease stage.

Table 5.1 Biomarkers specific to infection categories PS, IS, and AS, identified by primary peaks of PCA loadings, representative of the main biochemical differences between infected and control plants.

Infection Category	**Component**	**Wavenumber (cm^{-1})**	**Vibrational Mode**	**Biochemical Compound**	**References**
Pre-symptomatic (48 h PI)	PC-1 (65.6%)	1590 [a]	Amide I $\nu_{as}(COO)$ $\nu(C=N)$ $\nu(NH_2)$	Proteins Pectin (non-esterified) Lignin Nucleic acids	[5], [6], [8]
		1098 [a]	$\nu(C\text{-}O\text{-}C)$ ester $\nu(C\text{-}O)$ $\nu(C\text{-}C)$ $\nu_s(PO_4^{2-})$	Cutin Cellulose Pectin (as acetyl-ester) Polysaccharides DNA/RNA	[1], [2], [4], [5], [6], [8], [9]
		1061 [a]	$\nu(C\text{-}O)$ $\nu(C\text{-}C)$ $\delta(C\text{–}OH)$ $\delta(O\text{-}CH)$	Cellulose (particularly C_3-O_3H secondary alcohols) Polysaccharides Pectin Secondary metabolites (monoterpenes from leaf)	[1], [3], [5], [6], [9]
		995 [a]	$\omega(CH_2)$	Cellulose Secondary metabolites	[5], [7], [9]

			ν(C-O) ring stretching ν(C-C) ring breathing	(monoterpenes from fruit)	
	PC-2 (27.9%)	**1645**	Amide I (incl. secondary structure in β-sheet motifs) δ(O-H) ν(C=C)	Proteins Pectin Adsorbed water Secondary metabolites (ethylene, monoterpenes from fruit, sesquiterpenes)	[1], [2], [3], [6], [8], [9]
		1580	Amide I ν(C–C) ring stretch	Proteins Phenolic compounds	[7], [8]
		1092 [c]	ν(C-O) $\nu_s(PO_4^{2-})$	Pectin (also as acetyl-ester) Cellulose DNA/RNA	[1], [5], [8]
		975	$\nu(OCH_3)$ $\omega(CH_2)$ ω(RH-C-C-RH)	Pectin Protein phosphorylation Secondary metabolites (monoterpenes from leaf)	[6], [8], [9]
		1592 [a]	Amide I	Proteins	[5], [6], [8]

Intermediate Symptomatic (96 h PI)	PC-1 (74.0%)		ν_{as}(COO) ν(C=N) $\nu(NH_2)$	Pectin (non-esterified) Lignin Nucleic acids	
		1149 [d]	ν(C-O-C)	Pectin (ring and its glycosidic linkage) Non-cellulosic carbohydrates Arabinogalactan	[1], [5], [6], [9]
		1063 [a]	ν(C-O) ν(C-C) δ(C-OH) δ(O-CH)	Cellulose (particularly C_3-O_3H secondary alcohols) Polysaccharides Pectin Secondary metabolites (monoterpenes from leaf)	[1], [3], [5], [6], [9]
		1000 [a]	ν(C-O) ν(C-C) ν(O-C-H)	Cellulose Pectin	[1], [5], [9]
	PC-2 (10.9%)	**1748**	ν(C=O)	Pectin (also as ester) Polysaccharides Lipids Wax and suberin-like compounds Secondary metabolites	[1], [3], [5], [6], [9]

				(monoterpenes from leaf)	
		1592 [a]	Amide I ν_{as}(COO) ν(C=N) ν(NH_2)	Proteins Pectin (non-esterified) Lignin Nucleic acids	[5], [6], [8]
		1539 [d]	Amide II (incl. secondary structure in β-sheet motifs) ν(C=N) δ(NH_2)	Proteins Lignin Nucleic acids	[1], [3], [5], [7], [8]
		1105 [a]	ν(C-O) ν(C-C) ν_s(C-O-C) ester ν_s(PO_4^{2-})	Pectin (as acetyl-ester) Cellulose Polysaccharides Cutin DNA/RNA	[1], [2], [4], [5], [6], [8], [9]
		1038 [d]	ν(C-O) ν(C-C) ν(CCO) ν(O-CH_3) ν(CH_2OH)	Cellulose Pectin (also as acetyl-ester) Polysaccharides Xyloglucan Arabinogalactan Galactan	[3], [5], [8], [9]

Advanced Symptomatic (144 h PI)	PC-1 (63.2)	1600 [a]	ν(COO) ν(C=C) ν(C-C) ν(C=N) ν(NH_2)	Pectin Lignin Secondary metabolites (aromatic and phenolic compounds) Nucleic acids	[3], [4], [5], [6], [8], [9]
		1148 [d]	ν(C-O-C)	Pectin (ring and its glycosidic linkage) Non-cellulosic carbohydrates Arabinogalactan	[1], [5], [6], [9]
		1088 [c]	ν_s(C–O–C)	Pectin Xyloglucan Arabinogalactan Secondary metabolites (monoterpenes from leaves)	[5], [9]
		1063 [a]	ν(C-O) ν(C-C) δ(C–OH) δ(O-CH)	Cellulose (particularly C_3-O_3H secondary alcohols) polysaccharides Pectin Secondary metabolites (monoterpenes from leaf)	[1], [3], [5], [6], [9]

		996 [a]	ν(C-O) ring stretching ν(C-C) ring breathing	Cellulose Nucleic acids	[1], [5], [7]
	PC-2 (22.6%)	1598 [a]	ν(COO) ν(C-C) ν(C=C) ν(C=N) ν(NH_2)	Pectin Lignin Secondary metabolites (aromatic compounds) Nucleic acids	[3], [4], [5], [6], [8], [9]
		1537 [d]	Amide II (incl. secondary structure in β-sheet motifs) ν(C=N) δ(NH_2)	Proteins Lignin Nucleic acids	[1], [3], [5], [7], [8]
		1102 [a]	ν(C-O-C) ester ν(C-O) ν(C-C) $\nu_s(PO_4^{2-})$	Cutin Cellulose Pectin (as acetyl-ester) Polysaccharides DNA/RNA	[1], [2], [4], [5], [6], [8], [9]
		1037 [d]	ν(C-O) ν(C-C)	Cellulose Pectin (also as acetyl-ester)	[3], [5], [8], [9]

			ν(CCO) ν(O-CH_3) ν(CH_2OH)	Polysaccharides Xyloglucan Arabinogalactan Galactan	
		945	ω(CH_2)	Pectin Protein phosphorylation Secondary metabolites (monoterpenes from leaf)	[5], [8], [9]
Table References	[1] Abidi et al. 2014; [2] Butler et al. 2015; [3] Butler et al. 2017; [4] Heredia-Guerrero et al. 2014; [5] Largo-Gosens et al. 2014; [6] Monti et al. 2013; [7] Movasaghi et al. 2008; [8] Ord et al. 2016; [9] Schulz and Baranska 2007				
Bold wavenumbers represent unique spectral markers for each class. [a] : wavenumber overlap between all infection categories (PS, IS, and AS); [b] : wavenumber overlap between infection categories PS and IS only (not observed); [c] : wavenumber overlap between infection categories PS and AS only; [d] : wavenumber overlap between infection categories IS and AS only; ν: vibration; δ: deformation; ω: wagging.					

Vibrational modes at 1645, 1539, and 1038 cm^{-1} may contain absorbance peaks generated directly by the fungal pathogen, although these have not been assigned to nucleic acids previously (Salman et al., 2010, 2012). Contribution to absorbance at these wavenumber regions may come from fungal constituents including proteins (1645 and 1539 cm^{-1}) and chitin at 1038 cm^{-1} (Salman et al., 2010, 2012). The ATR-FTIR data represents the complex interaction of tomato leaf tissue and fungal mass as a mixture effect and further research will determine in detail the contributions of each organism to the observed spectral changes. A small fraction of wavenumbers 1748, 1645, 1580, 975, and 945 cm^{-1} were unique either to PS, IS, or AS. Categorically, 1645, 1580, and 975 cm^{-1} were unique markers to the PS disease stage, while 1748 and 945 cm^{-1} were associated with IS and AS, respectively (Table 5.1). In contrast to IS and AS stages, the PS stage showed the highest number of unique markers, compared to only one for both IS and AS stages. This is consistent with the higher degree of variance explained for PS along PC1 and PC2 compared to IS and AS plants. While PS markers covered both upper (1645 and 1580 cm^{-1}) and lower (975 cm^{-1}) regions of the fingerprint spectrum, IS showed unique absorbance only at 1748 cm^{-1}, whereas AS was uniquely identified by absorbance at 945 cm^{-1}. Unique fingerprint changes in the PS stage were associated with changes in the UF and carbohydrate regions, specifically the Amide I region of proteins (1645 and 1580 cm^{-1}) and polysaccharides associated with pectin (975 cm^{-1}) (Abidi et al. 2014; Butler et al. 2015; Butler et al. 2017; Monti et al. 2013; Ord et al. 2016; Schulz and Baranska 2007). Alternatively, these regions can also be related to changes in phenolic compounds of secondary metabolites, and protein phosphorylation (Heredia-Guerrero et al. 2014; Ord et al. 2016; Schulz and Baranska 2007). The IS stage showed a single unique wavenumber at 1748 cm^{-1}. This wavenumber corresponds predominantly to changes in carbohydrates and or lipids, including pectin and waxes, likely reflecting substantial changes in plant epidermal layers such as the cuticle and cell wall (Abidi et al. 2014; Butler et al. 2017; Largo-Gosens et al. 2014).

Onset of visual symptoms, at both IS and AS, are likely linked to processes including programmed cell death (PCD) and tissue necrosis (Dieryckx et al. 2015; Hoeberichts et al. 2003). At the other extreme of the fingerprint spectrum, a vibrational mode at 945 cm^{-1} is a unique spectral biomarker to the AS stage. Compounds related to this wavenumber include pectin, protein phosphorylation, and secondary metabolites, plausibly reflecting continued pectin degradation, alterations in protein structure through covalent modifications (phosphorylation), and or changes

in secondary metabolite production, as a response to the necrotrophic mode of *B. cinerea* (Largo-Gosens et al. 2014; Ord et al. 2016; Schulz and Baranska 2007).

Determining the exact identity of IR compounds and their related processes require replicate experiments, as currently available methods do not permit reliable biochemical validation of entities exactly, which remains a significant drawback of spectroscopy-based approaches. For this reason, identifying target compounds relevant for plant-pathogen interactions with necrotrophic fungi is a vital first step in pathogen detection and generating disease specificity based on spectral biomarkers, especially within whole-plant systems using MIR spectroscopy.

5.3.3 Detecting Disease Progression Directly by Discrimination and Classification of Infection Category

To determine the effectiveness of direct pathogen detection based on plant tissue modifications, classification algorithms may be especially useful as they do not rely on the validation of biochemical entities to be effective. This dual approach, combining exploratory with diagnostic data analysis, provides data that may help answer specific biological questions as well as immediate discriminatory information for detecting plant disease autonomously (Kelly et al. 2011; Trevisan et al. 2012). Direct detection of plant infection throughout disease progression can be achieved autonomously by combining spectral data with multivariate analysis and machine classifiers. Biochemical information in the form of MIR spectra has been combined previously with supervised multivariate analysis and diverse classification algorithms to assess computer-based decision-making for autonomous diagnostics performance using MIR spectral input for disease detection in animal models (Martin et al. 2007; Morais and Lima 2017; Trevisan et al. 2012). Complete class separation was attained for PS, IS, and AS plants after exposure to *B. cinerea*; Figure 5.4 shows PCA-LDA scores plots and clear separation of healthy plants from PS (A), IS (B), and AS (C) across DF1. Based on the complete data separation observed along DF1, diagnostic potential for plant disease detection was evaluated via classifier output (Table 5.2). PCA-LDA classification accuracy was equal to 100% in the validation set and was thus highly effective for disease detection for all groups (PS, IS, AS). All groups of plants were classified with 100% sensitivity and specificity. The models were trained with 4 PCs (Table 5.S2), having a training accuracy of 100% for all types of infection comparisons (Table 5.2), and a cross-validation accuracy of 98% for IS and 100% for both PS and AS (Table 5.S3).

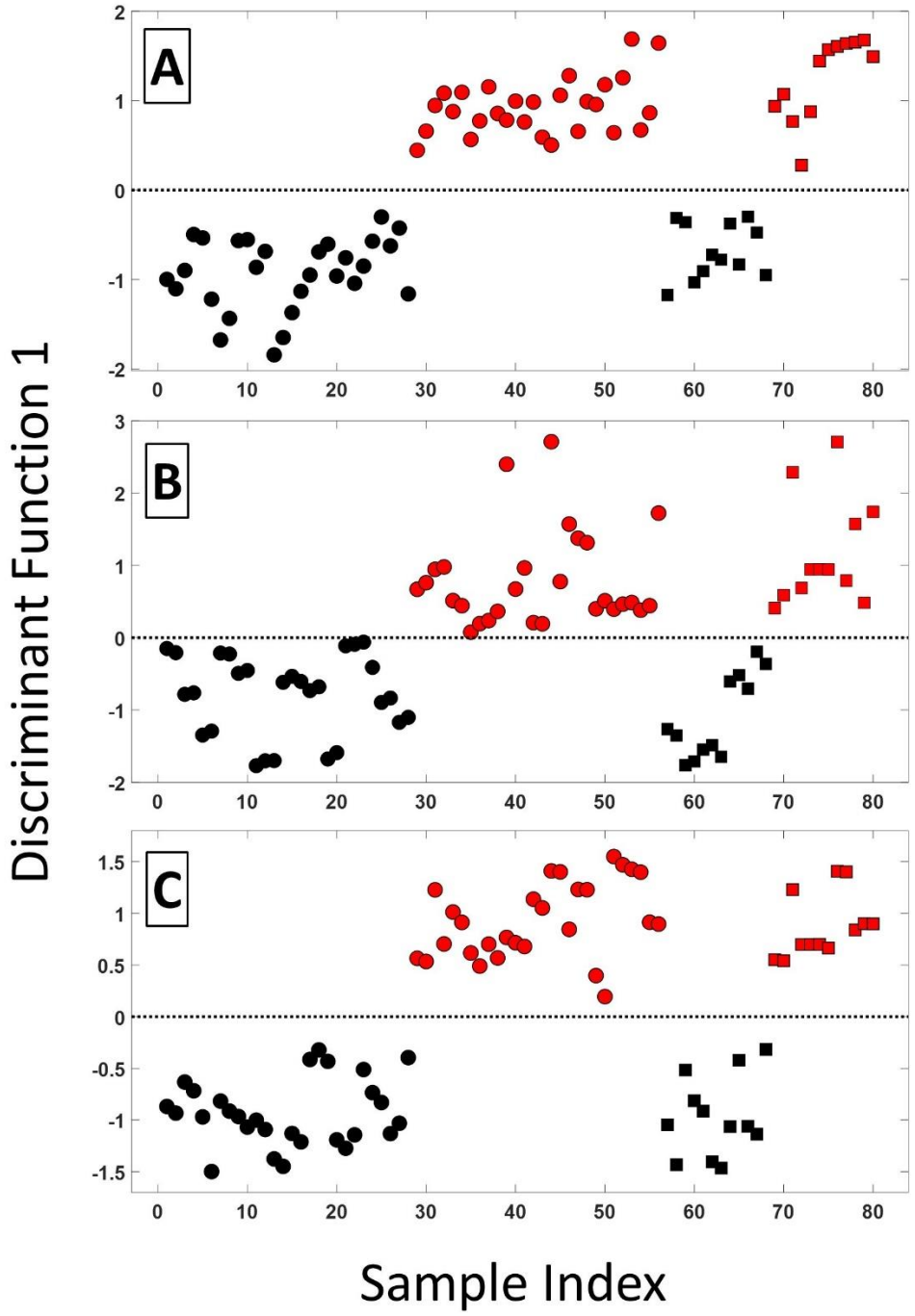

Figure 5.4 Cross-validated PCA-LDA scores plots of pairwise control (black) versus *B. cinerea* infected (red) tomato leaf spectra at 48 (A), 96 (B), and 144 (C) h. Training data (circles); validation data (squares).

Table 5.2 Validation set classification results of control versus infected tomato plants using PCA-LDA.

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

Perfect classification rates strongly suggest that biospectroscopy, combined with chemometric classifiers, offers a suitable approach to the development of diagnostic tools for plant disease. Importantly, the accurate classification of PS plants demonstrates that early identification of *B. cinerea* infection can be achieved in completely intact tomato plants, prior to the onset of symptoms on which current diagnostics approaches rely. Therefore, detection of PS plants is especially valuable, because the PS disease stage offers a longer window for the application of crop protection measures, which are becoming part of specialized modern approaches including integrated pest management (IPM) and precision crop protection (Barzman et al. 2015).

5.4 Conclusions and Perspectives

MIR biospectroscopy is a capable technology applicable to the development of precision sensors aimed at mitigating crop loss due to pests and pathogens. However, although biospectroscopy presents a novel analytical technique, it has as yet been little used in the plant and crop sciences, especially for applications to dynamic intact plant-pathogen systems (Skolik et al. 2018a). Here it was demonstrated that biospectroscopy in the form of semi-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 5.1). Clear differences in the class mean spectra were observed primarily in the UF and carbohydrate regions (Figure 5.2). This was consistent with specific spectral biomarkers extracted via multivariate analysis by way of cluster analysis and through PCA loadings (Figure 5.3 and Table 5.1). 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). 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 5.4 and Table 5.2). With a classification accuracy of 100%, this approach appears highly suitable for pre- and post-symptomatic disease detection.

Rapid sensor-based disease detection will contribute to reductions in 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. However, further research is required with respect to MIR biospectroscopy-based disease detection to increase the range of studies performed on intact plants *in vivo* focusing on model plants/crops, as well as the evaluation of portable equipment suitable for the field (Skolik et al. 2018b). 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; Farber 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 (Skolik et al. 2018b). 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.

5.5 Supplementary Material

Table 5.S1 *B. cinerea* infected tomato and observed symptomatic used for ATR-FTIR analysis (corresponding to Figure 5.1)

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: yellowing and large lesions covering >50% of leaf area, necrotic and or desiccated tissue, visible hyphae, pathogen sporulation.

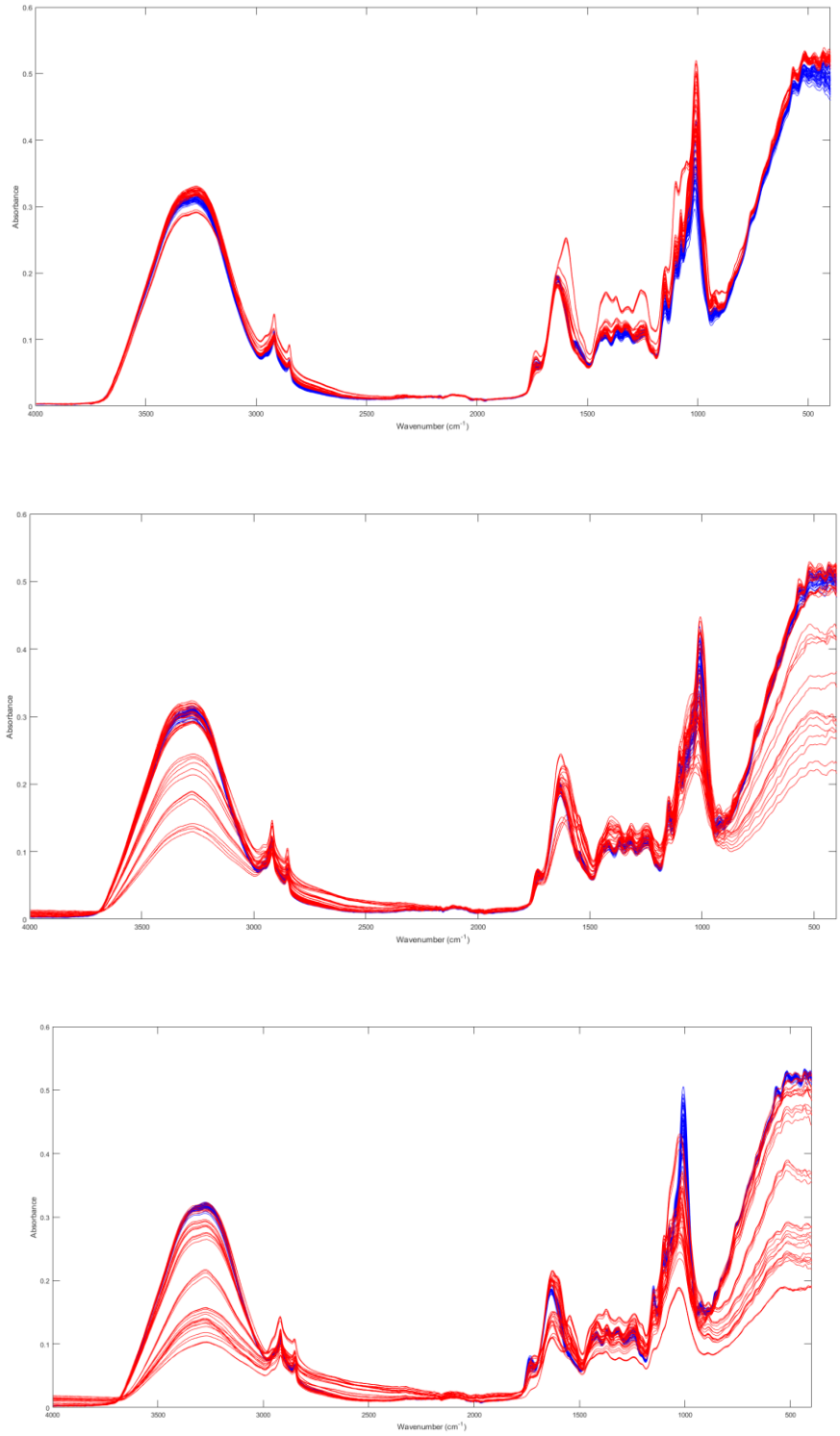

Figure S5.1 Raw spectral data for pairwise comparisons at 48 (PS), 96 (IS), and 144 (AS) h (blue: controls; red: infected).

Table S5.2 Principal component analysis (PCA) explained variance for each infection category.

Principal Component	Pre-symptomatic		Intermediate Symptomatic		Advanced Symptomatic	
	Variance	Cumulative Variance	Variance	Cumulative Variance	Variance	Cumulative Variance
1	65.52%	65.52%	73.97%	73.97%	63.18%	63.18%
2	27.96%	93.48%	10.92%	84.89%	22.54%	85.72%
3	3.86%	97.34%	7.23%	92.12%	8.36%	94.08%
4	1.62%	98.96%	3.34%	95.46%	2.45%	96.53%

Table S5.3 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	2%	98%
144 h (AS)	Control	100%	0%
	Infected	0%	100%

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Chapter 6 Discussion and Conclusions

6.1 Discussion

Rapid identification of plant disease in the field, caused by PPs, remains a formidable challenge. Numerous approaches are available to manage plant PPs in crops, that are based on different aspects of the plant-pathogen interaction (Barzman et al. 2015). From an IPM approach, PPs need to be managed at various times throughout the growth of the crop, depending on their distribution and abundance in crop systems (Barzman et al. 2015). This ranges from detection of PPs in growing mediums prior to planting, to managing compromised post-harvest crop products such as damaged or decaying fruit (Skolik et al. 2018b; Van Gent-Pelzer et al. 2010). There are many sensor technologies available which are potentially of use for the detection of PPs. Traditional approaches have focused on late-stage detection using genetic confirmation using polymerase-chain reaction (PCR) or serological assays (Martinelli et al. 2014). Many of these specialized approaches however, are based on complex plant biology and are in the experimental phases and therefore their use is limited by either the need for experts with the appropriate technical experience or because they are not easily implemented under highly variable field conditions (Mahlein 2016; Martinelli et al. 2014; Sankaran et al. 2010; Skolik et al. 2018b). The primary sensor types under evaluation currently are molecular techniques including (PCR) and enzyme linked immuno-sorbent assay (ELISA), VOC sensors, and light-based sensors (bio-photonics, spectroscopic, hyperspectral, remote sensing, etc.) (Martinelli et al.2014; Sankaran et al. 2010).

While the principal aim of such experimental methods is to detect the early onset of plant disease, this is especially difficult with microscopic PPs, the presence of which is not readily observed. Consequently, microscopic PPs remain difficult to detect and manage (Williamson et al. 2007; Bebber 2015). Analytical techniques that are both robust and rapid enough to be employed for purposes of disease management, specifically for detecting PP effects on crop plants pre-symptomatically in the field, are therefore desperately needed (Skolik et al. 2018b). The research reported in this thesis investigates the potential of MIR vibrational biospectroscopy to detect plant disease at various stages from pre-symptomatic through disease progression pre- and post-harvest. Attention was focused on the extraction of biochemical information from intact crops in the form of spectral biomarkers, in order to begin to develop disease-specific spectral biomarkers, while concurrently evaluating machine learning-based approaches for autonomous decision-making systems based on spectral data. This combined approach is examined throughout the research in

order to evaluate the potential of developing non-destructive autonomous detection of PPs for commercial applications in food production and in the supply chain.

The application of MIR biospectroscopy in plant and crop science requires the establishment of the fundamental principles required to advance these methodologies for intact plant analysis and early disease detection. Chapter 2 considers the development of this approach as a horticultural sensor and applications focused on intact crop plants and thus *in vivo* analysis, applicable to the detection of PPs in crops and environmental interactions in general (Skolik et al. 2018b). This chapter explores the primary components involved in biospectroscopy studies, including sample preparation, spectral acquisition, and computational analysis. These concepts apply to the complementary methods MIR as well as Raman spectroscopy (Skolik et al. 2018b). In particular, the issue of non-destructive sample analysis and *in vivo* analysis of plant tissues is discussed. The application of biospectroscopy to fundamental plant research and crop science is considered with regards to the identification and classification of valuable plant components and identifying specific species or cultivars, for which destructive sample preparation is acceptable or even necessary (Schulz and Baranska 2007,). For *in vivo* analysis however, a major focus is the analysis of intact plant tissues, more specifically those that can be interrogated by MIR and Raman lasers (Baker et al. 2014; Butler et al. 2016). These include the plant epidermis and fundamental surface structures contained therein, such as the cuticle and cell wall (Figure 2.2). Therefore, plant surface structures including the cuticle and cell wall are emphasized primary contributors to the plant spectral fingerprint.

For a true biological interpretation of dynamic processes (biochemistry / physiology) occurring within plants, analysis must be performed on the native tissue architecture *in vivo*. If this is the case, then the plant epidermis becomes the main target of biospectroscopy approaches. Plant epidermis can then be used to investigate plant-environment interactions, which include the interaction between plants and PPs *in vivo* (Egging et al. 2018; Skolik et al. 2018a). The limited number of studies in this area to date demonstrate that *in vivo* analysis of intact plant materials is not yet routine but certainly possible with both ATR-FTIR and Raman spectroscopy (Butler at al. 2015; Skolik et al. 2018a).

Parameters of biospectroscopy methods, specifically sample preparation, spectral acquisition, and data analysis are discussed, in the context of moving towards more *in vivo* measurements and thereby gaining more biologically relevant information. In addition,

recommendations are made as to how to advance 'whole-plant' MIR biospectroscopy for researchers new to MIR biospectroscopy of plants. Among these recommendations are specific considerations including portable equipment, measurement area of the laser and penetration depth into the sample. It was determined, based on this research, that macro-FTIR measurements were more suitable for the interrogation of tissue sections on the whole-plant level than previously used Raman or micro-FTIR measurements (Egging et al. 2018; Farber and Kurouski 2018; Yeturu et al. 2016). The popularity of Raman spectrometers is because they are currently available in a more portable format compared to the more suitable but less portable ATR-FTIR spectrometers (Skolik et al. 2018b). MIR spectroscopy measurements in the field are arguably impeded by increased sensitivity to water and thus humidity compared to Raman spectroscopy, which is not influenced by excess water (Butler et al. 2019; Maréchal 2011). Due to the better performance of ATR-FTIR in macro mode, this was therefore used as the standard throughout, although where possible, portable spectrometers were used to compare their performance to bench-top instruments and thereby facilitate the transition of lab-field based measurements.

To promote the transition of these approaches from the laboratory into field environments, portable macro ATR-FTIR was chosen for non-destructive crop analysis in fruit of *S. lycopersicum* during development and ripening (Chapter 3). This provided a complementary approach to baseline characterization of healthy fruit, which had previously been established in whole plants of *S. lycopersicum* (Butler et al. 2015). Taken together, these studies provide baseline spectral signatures for both tomato plants and fruit during development from young plants through to maturity, fruit development and ripening all using ATR-FTIR spectroscopy (Figure 3.2-3.6 and Table 3.1 and 3.2). Previous studies related to development and ripening of intact tomato fruit, using portable Raman spectroscopy have been performed (Fu et al. 2016; Trebolazabala 2013, 2017). These studies, aside from using Raman compared to MIR spectroscopy, focused on other aspects of tomato fruit development/ripening and not specifically the biology of both processes.

To address this, development and ripening were measured over time at intervals of 4 days, providing nine developmental stages and six ripening stages for analysis (Skolik et al. 2019). Multivariate analysis provided a summary of spectral biomarkers for the development and ripening processes, as these are often investigated separately. Furthermore, spectra of fruit were input for classification with SVM leading to accuracy of more than 99% for autonomous monitoring of development and ripening (Table 3.3 and 3.4). While similar accuracy rates have been reported

for tomato fruit, considerably lower numbers of classes were used, or dealt with only one of the developmental or ripening processes (Fu et al. 2016; Trebolazabala et al. 2013, 2017). Expanding on the previous studies, a more detailed study was performed, where the developmental timeframe was divided into nine classes; ripening was split into six groups as these were horticulturally relevant (Figure 3.1). Spectral data including compounds tagged as spectral biomarkers (Table 3.1 and 3.2), advance the biochemical understanding of *S. lycopersicum* fruit development/ripening from a spectrochemical perspective, which could be combined with other sensors and biochemical assays to elucidate the molecular details of specific changes to the cuticle and cell wall (Egging et al. 2018; Skolik et al. 2018b). Additionally, this research provides a more detailed spectral analysis of both development and ripening than previously performed, providing information that can be integrated with other data as more is learned about the molecular biology of these processes (Segado et al. 2016; Seymour et al. 2013). Classifier performance may be developed for detailed crop screening and the potential identification of abnormalities to ensure high post-harvest quality of fruits and prevent disease induced crop loss (Skolik et al. 2018a). However, it is first essential to characterize changes in the spectral fingerprint during the development and ripening of tomato fruits prior to investigating effects caused by environmental factors including PPs in order to ensure that biomarkers indicative of disease can be distinguished from those associated with normal developmental and biochemical/physiological processes.

Following baseline characterization of whole tomato fruit in Chapter 3, ATR-FTIR spectroscopy was used to investigate the potential of this approach for studying whole tomato cultivars exposed to PPs. Chapter 4 presents research illustrating the application of ATR-FTIR spectroscopy for the non-destructive detection, both directly and indirectly, of PP effects on fruit. Although previous studies have shown direct pathogen detection within plant tissues (Egging et al. 2018; Yeturu et al. 2016), it has been difficult to detect the effects of pathogens on fruit indirectly. Thus, the demonstration of both direct and indirect detection potential in the same system represents a significant stride towards elucidating the spectrochemical changes associated with biochemical mechanisms in tomato fruit disease. It was first shown that the spectral fingerprint of plant tissue is altered at sites distant from the actual infection and initial damage, where no direct damage or infection was present (Figure 4. 3). Conceptually this can be extended to investigate the indirect effects of PPs on plant tissues that are removed from the site of insult. Additionally, the demonstration that damage can be used as a pre-symptomatic indicator of disease

or of fruit decay potential caused by opportunistic organisms such as the investigated *G. candidum* represents an exciting advance (Skolik et al. 2018a). Direct detection of the fungal pathogen *G. candidum* on tomato fruit following damage showed significant spectral alterations to the fungal signature when forming a complex with tomato fruit *in vivo* (Figure 4.4). Assessment of damage and decay using more portable equipment to facilitate field-based measurements would be the next steps to implementing biospectroscopy commercially.

Limitations to this approach is the external validation of this technique using a real-life sample set. Even though the experimental data generated was on real-world samples with no sample preparation, it was not strictly performed under industry conditions, where datasets would be much larger and measurement conditions more variable. Larger datasets would likely increase the detection accuracy, while variable measurement conditions may offset this performance by requiring more optimized modeling before commercial implementation. Nevertheless, is highly likely that the demonstrated results translate and be achieved in practice (Farber and Kurouski 2018). Future work will include the assessment of damage and decay using more portable equipment to facilitate field-based measurements (Egging et al. 2018).

The ability to detect PP-induced disease in whole plants was assessed in young tomato plants infected with grey mould (*B. cinerea*) (Chapter 5). Although disease detection was effective at all stages of infection studied, both pre- and post-symptomatic disease stages, the pre-symptomatic stage was the most interesting in that early detection was effective prior to the onset of any visual symptoms (Skolik et al. 2019). This contrasts with studies requiring extensive sample preparation (Farber and Kurouski et al. 2018), experts for visual determination of symptoms (Egging et al. 2018) or relying on pre-symptomatic leaves from clearly symptomatic plants and therefore requiring cutting (Yeturu et al. 2016), which as described in Chapter 2 is destructive sample preparation. Thereby, the research presented in Chapter 5 represents the pre-symptomatic condition more accurately, under analysis conditions that do not alter the native state of the plant (Figure 5.1).

Plants infected with *B. cinerea*, were exposed uniformly to the pathogen, making it possible to show direct pathogen detection potential only. As all plant parts measured were exposed and potentially contained fungal material, it was not possible to rule out the direct contribution of this to the spectral fingerprint. During disease, plant and pathogen can be seen as a mixture matrix containing both plant and pathogen tissues (Figure 5.1). Thus, direct detection will generally be

the norm for future sensor systems (Egging et al. 2018; Farber and Kurouski et al. 2018; Yeturu et al. 2016). Importantly, Chapter 5 focused on whole-plant analysis addressing the pre-symptomatic stage in greater detail than has been done previously. Discriminant biomarkers and class separation between infected and healthy plants were able to be produced using PCA alone (Figure 5.3 and Table 5.1), making PCA-LDA highly effective. This was shown with classification accuracy of 100% for all stages of disease (Table 5.2).

It is likely that the indirect disease detection achieved on fruit (Chapter 4) can be translated to plant analysis for pre-harvest applications. Cuticle and cell wall components were identified as discriminating compounds in spectral fingerprints of fruit during development, ripening, damage, and infection by *G. candidum*, and leaves infected with *B. cinerea*. It is therefore plausible that the spectral biomarkers identified tentatively as part of this research (see biomarker tables in Chapters 3-5 for specific compound classifications), or a subset of these will be confirmed by further research as markers ultimately used in the field. This is because surface structures described in Chapter 2 are key biological barriers involved in plant-environment interactions, which are general biospectroscopy targets (Skolik et al. 2018b). As these epidermal structures are features of both plants and leaves, it is expected that biospectroscopy will be equally applied to pre and post-harvest systems.

The ATR-FTIR approach demonstrated here on whole tomato plants infected with *B. cinerea*, complements similar spectroscopic approaches shown previously on whole crops, which provides the basis for a multi-sensor approach to disease detection (Mahlein 2016). Potential future applications of this approach range from disease detection to monitoring and studying disease progression including some of the major biochemical changes happening in tomato plant tissue infected with fungal pathogens. These studies therefore provide the initial data upon which to develop biospectroscopy-based approaches to reduce the effects of pathogens as well as damage on crop loss involving tomato fruit.

6.2 Conclusions

Advancement of biospectroscopy as a sensor technology for application in the plant and crop sciences is progressing rapidly (Butler et al. 2015; Canteri et al. 2019; Herredia-Guerrero et al. 2014). These methods are sustainable, non-destructive, fast, and precise for *in vivo* analysis of crop plants pre and post-harvest (Canteri et al. 2019; Skolik et al. 2018b). Furthermore,

biospectroscopy is adaptable to a variety of crops and plant-pathogen systems (Canteri et al. 2019; Egging et al. 2018; Farber and Kurouski et al. 2018). The experimental data presented in this thesis indicates that vibrational spectroscopy can generate high quality spectra of intact plant leaves and fruit, demonstrating greenhouse applicability which is likely to be translated to the field in the near future. Analysis without sample preparation is readily achieved using this approach, increasing the applicability of these techniques to real-world situations. ATR-FTIR performed well under semi-controlled greenhouse conditions (Skolik et al. 2019), meaning that translation into the field in the form of portable instruments is likely, if the limitation of water sensitivity can be overcome.

Both baseline characterization and the successful pathogen detection in the model system *S. lycopersicum* highlights the discriminatory power of spectral data for whole plant analysis in various scenarios, demonstrating clearly the potential for pest detection (Chapter 4 and 5). Multivariate analysis provides a novel approach for identifying the biochemical/physiological changes related to the observed alterations in spectra, caused by healthy growth and development or by disease. Specifically, alterations in the spectral signatures (Chapter 3-5) were due to biological processes occurring in epidermal tissues of fruit and leaves that were the primary MIR targets in intact crops. The early detection of plant-pathogen interactions at the pre-symptomatic stage, which was a major objective of the research, was demonstrated effectively under pre- and post-harvest conditions in chapter 5 and chapter 4 respectively. This important result demonstrates the potential from MIR spectroscopy to inform crop protection measures that can be applied early before adverse effects become apparent. Once adapted for commercial use, biospectroscopy has the potential to significantly reduce crop loss in the future.

6.3 Future Perspectives

The future of biospectroscopy is bright in the plant and crop sciences, as the current trend of increasing applications of IR spectroscopy in biology continue. More than half a century ago, infrared vibrational spectroscopy became of increasing interest for the investigation of biological systems (Mantsch and Shaw 2002). As Shaw and Mantsch (1999) put it at the turn of the century, “The idea of using vibrational spectroscopy to explore the properties of living organisms is as absurd as it is compelling”. Today however, continued display of the applicability of biospectroscopy to *in vivo* systems suggests a possible paradigm shift to include routine analysis of active processes, making this approach less ‘absurd’ and more ‘compelling’ than ever before.

As part of this continued expansion in the plant and crop sciences, several key issues remain to be addressed (Skolik et al. 2018b). Validation and standardization of data analysis and either development of customized platforms for difficult plants/crops or frameworks adaptable to diverse conditions, crops, and cultivars. High throughput biospectroscopy approach with respect to minimal sample preparation (whole plants and plant products) and continued studies *in vivo* such that spectral data obtained retains biological relevance. Optimization of spectral acquisition, specifically the number of spectra required for adequate classification and reproducible spectral-biomarker identification. Continuing studies using portable equipment and comparing the quality of spectral data to bench-top or lab-based instruments. Retooling and implementing such spectroscopic equipment for field use is another practical and final challenges, granting various portable instruments are already available. Development of computational analysis methods: extraction of biomarkers and identification of data analysis models generating appropriate disease specificity extracted using chemometrics.

Biospectroscopy will therefore contribute to deciphering the many, yet unknown, processes within the realm of fundamental plant biology, which will be translated, through knowledge-exchange, to building a sustainable and modern farming. With the exceptionally rapid evolution of MIR biospectroscopy to date, come countless opportunities to further apply these methods to virtually any biological system, whether static or dynamic, but it is the latter of these that will prove to push the boundaries of this field in the coming future.

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Appendix I: Academic Activities

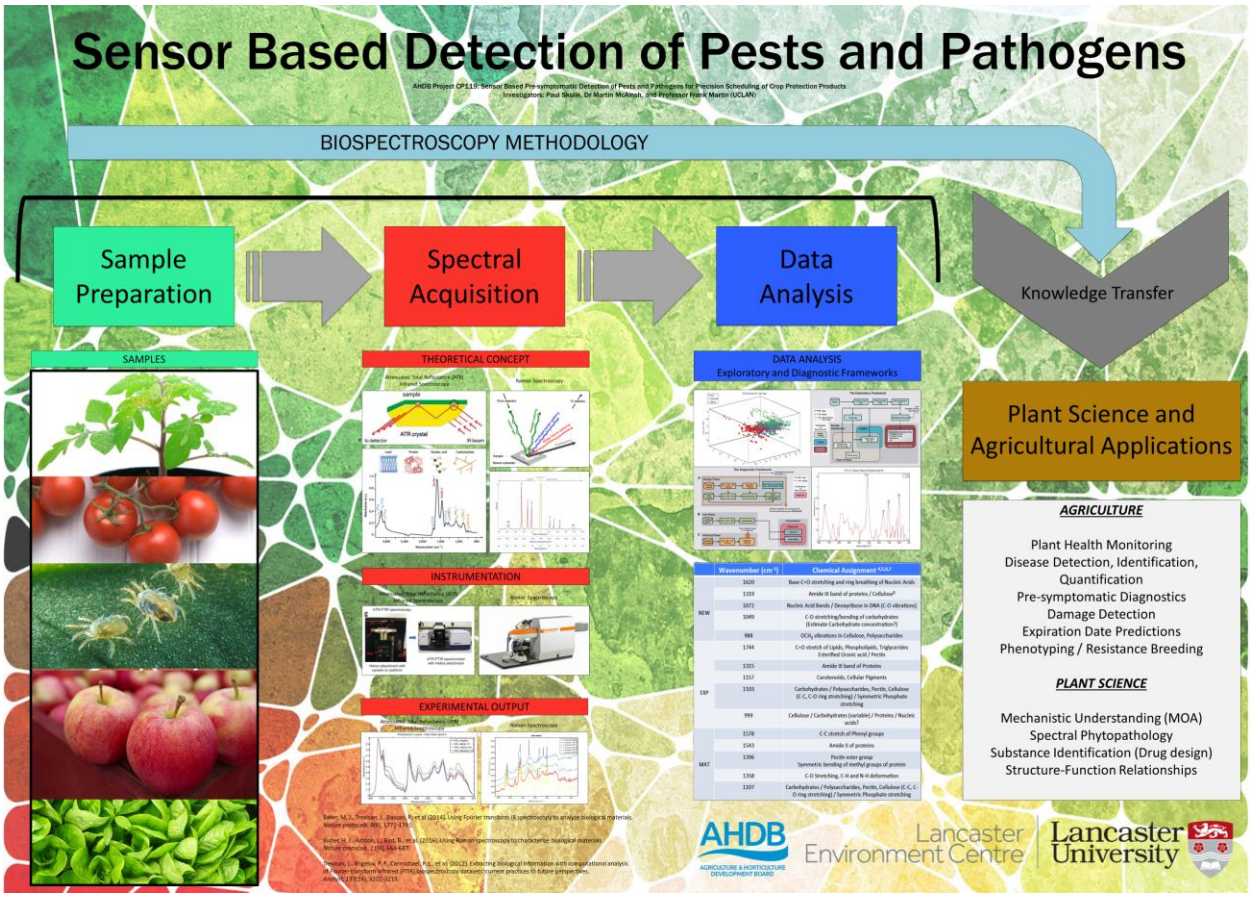
Conference Contributions

Exploiting novel sensors for detecting abiotic and biotic stress in crops Contribution: 20-minute oral presentation	June 2016
Lancaster Environment Center Postgraduate Research Conference Contribution: 15-minute oral presentation / Poster presentation	July 2016
AHDB PhD Student Conference Contribution: Poster	November 2016
Lancaster Environment Center Christmas Conference Contribution: 15-minute oral presentation	December 2016
Lancaster Environment Center Postgraduate Research Conference Contribution: 15-minute oral presentation	July 2017
AHDB PhD Student Conference Contribution: 10-minute oral presentation	November 2017
University of Central Lancashire (Laboratory Meeting) Data Review Contribution: 30-minute oral presentation	March 2018

Other Activities

AHDB Industry Visits	2015-2018
Daresbury Laboratory Research Associate (High Energy Laboratory)	2015 2016-2018
Lancaster Environment Center Associate Lecturer	2015-2019
Brilliant Club Tutor	

Appendix II: Conference Posters



BIOSPECTROSCOPY

In Plant Science and Horticulture

CP119: SENSOR BASED PRE-SYMPTOMATIC DETECTION OF PESTS AND PATHOGENS FOR PRECISION SCHEDULING OF CROP PROTECTION PRODUCTS

PhD Student: Paul Skolik

Supervisors: Dr Martin McAinsh and Professor Frank Martin

AHDB AGRICULTURE & HORTICULTURE DEVELOPMENT BOARD

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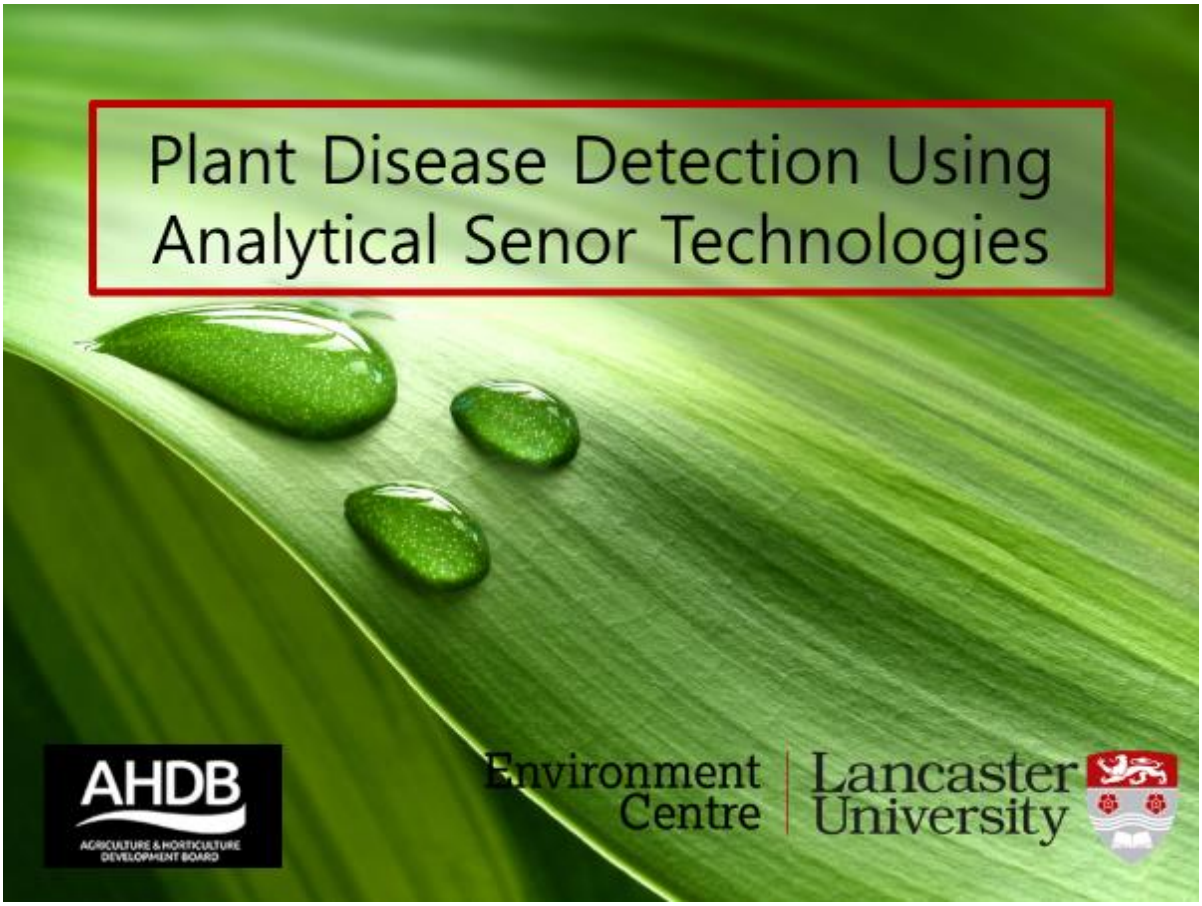

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FOR PLANT AND CROP SCIENCE

Lancaster Environment Centre | Lancaster University

AHDB Project CP:119 Sensor Based Pre-symptomatic Detection of Pests and Pathogens for Precision Scheduling of Crop Protection Products