Journal of Photochemistry & Photobiology, B: Biology 180 (2018) 155–165

Contents lists available at ScienceDirect

Journal of Photochemistry & Photobiology, B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

Differentiation of mixed soil-borne fungi in the genus level using infrared spectroscopy and multivariate analysis

M. Huleihel[a,*], E. Shufan[b], L. Tsror[c], U. Sharaha[a], I. Lapidot[d], S. Mordechai[e], A. Salman[b,*]

[a] Department of Microbiology, Immunology and Genetics, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel
[b] Department of Physics, SCE-Sami Shamoon College of Engineering, Beer-Sheva 84100, Israel
[c] Department of Plant Pathology, Institute of Plant Protection, Agricultural Research Organization, Gilat Research Center, M.P. Negev 85250, Israel
[d] Department of Electrical and Electronics Engineering, ACLP-Afeka Center for Language Processing, Afeka Tel-Aviv Academic College of Engineering, Israel
[e] Department of Physics, Ben-Gurion University, Beer-Sheva 84105, Israel

ARTICLE INFO

Keywords:
FTIR-microscopy
Colletotrichum
Verticillium
Rhizoctonia
Fusarium
PCA
LDA

ABSTRACT

Early detection of soil-borne pathogens, which have a negative effect on almost all agricultural crops, is crucial for effective targeting with the most suitable antifungal agents and thus preventing and/or reducing their severity. They are responsible for severe diseases in various plants, leading in many cases to substantial economic losses. In this study, infrared (IR) spectroscopic method, which is known as sensitive, accurate and rapid, was used to discriminate between different fungi in a mixture was evaluated. Mixed and pure samples of *Colletotrichum*, *Verticillium*, *Rhizoctonia*, and *Fusarium* genera were measured using IR microscopy. Our spectral results showed that the best differentiation between pure and mixed fungi was obtained in the 675–1800 cm^{-1} wavenumber region. Principal components analysis (PCA), followed by linear discriminant analysis (LDA) as a linear classifier, was performed on the spectra of the measured classes. Our results showed that it is possible to differentiate between mixed-calculated categories of phytopathogens with high success rates (~100%) when the mixing percentage range is narrow (40–60) in the genus level; when the mixing percentage range is wide (10–90), the success rate exceeded 85%. Also, in the measured mixed categories of phytopathogens it is possible to differentiate between the different categories with ~100% success rate.

1. Introduction

Soil-borne fungal pathogens are responsible for severe diseases in various crops leading to substantial economic damage including yield loss, decreased quality of the plant or plant product and cost of control [1,2].

Plants are often infected with different pathogens simultaneously with similar symptoms. For instance, potato may be infected at different growth stages by various fungi, *e.g. Fusarium oxysporum*, *Verticillium dahliae*, *Colletotrichum coccodes* causing wilt and plant early dying. In such situations, the diseases cannot be identified based on visual symptoms alone.

In previous studies [3–5], we have demonstrated the potential of infrared spectroscopy together with pattern recognition methods, as a tool for differentiation between different types of fungi. However, in many cases a mixture of more than one fungus that causes similar symptoms may be involved in the same infection.

In this study, mixed samples of *Colletotrichum*, *Verticillium*, *Rhizoctonia*, and *Fusarium* phytopathogens were investigated in the genus level. Early detection and accurate identification are crucial for effective control of these pathogens [6]. It is very important to identify all associated pathogens, in order to choose the best antifungal agents and treatments. Commercially available methods are based on morphological, biochemical or physiological characteristics. Although these methods are inexpensive they are time consuming (often taking weeks to complete) [7], they have low specificity [8–10] and they are not effective in identification of species or strains [11]. Other methods include serological tests based on the specificity of the antigen-antibody reaction in immune systems, but they are not always highly specific. Molecular based methods such as PCR (polymerase chain reaction), restriction fragment length polymorphism (RFLP) and sequencing strategies such as multilocus sequence typing (MLST) are considered as the most sensitive and specific methods, however, these methods are time consuming and expensive [12]. Therefore, development of a cheap method for identification of soil-borne pathogen infestation prior to planting would enable more precise and selective application of control measurements.

Infrared spectroscopy is being increasingly examined for its

* Corresponding authors.
E-mail addresses: mahmoudh@bgu.ac.il (M. Huleihel), ahmad@sce.ac.il (A. Salman).

https://doi.org/10.1016/j.jphotobiol.2018.02.007
Received 31 May 2017; Received in revised form 24 January 2018; Accepted 6 February 2018
Available online 07 February 2018
1011-1344/

potential as a diagnostic tool for biological samples and as a reagent-free and rapid method. Many studies have shown that FTIR spectroscopy, in tandem with multivariate analysis, became an excellent method with a significant potential to identify and study microorganisms, even in the isolate level. The biochemical changes in the cellular and sub-cellular levels of different phytopathogens can be detected using infrared spectroscopy. The molecular vibrational modes are reflected in the IR absorption spectra, and are characteristic of the biochemistry of the cells and their sub-cellular components.

For microbiological identification, both supervised and unsupervised methods can be used in chemometric techniques [3–5,13–16]. Naumann et al. [17] have shown that FTIR absorption spectra are highly specific fingerprints of microbial cells and that by using IR spectroscopy it is possible to differentiate between bacterial cells, even in the isolate level.

Although the potential of the ATR sampling technique has not been evaluated for *in-situ* detection of infected foodstuffs, it has good chances for this purpose similar to what has been done in previous studies [18].

The use of FTIR spectroscopy followed by PCA-LDA multivariate analysis for biological classification has gained momentum in recent decades [4,5,14,17–20]. Indeed, the combination of PCA-LDA has been proven as a robust method for the identification of different biological systems as demonstrated by several groups [21–31].

The present work evaluates *in-vitro* the potential of FTIR microscopy methodology in tandem with multivariate analysis to classify mixed samples of four different fungi genera: *Colletotrichum*, *Verticillium*, *Rhizoctonia*, and *Fusarium*. The samples were prepared by mixing different pairs of these genera. In addition, we evaluated to what extent this method is sensitive to mixing percentage levels of different fungi present in the sample.

2. Material and Methods

2.1. Fungi

All examined fungi isolates (Table 1) were supplied by the Department of Plant Pathology at the Gilat Experiment Center, ARO, Israel. In this study, 1054 different pure samples of four different soil borne fungi of the genera *Colletotrichum*, *Verticillium*, *Rhizoctonia*, and *Fusarium* and 488 mixed samples (in couples of these four genera) were measured as detailed in Table 1. At least five strains were included for each genus. At least 230 samples were measured from each genus. The samples of each genus came from at least 20 different initial fungal sources (from the field) and from each of these 20 sources we created > 10 further different samples. Each spectrum was in fact obtained from a different batch of the fungus.

2.2. Sample Preparation

As a first step, the fungi were cultivated and identified using classical microbiological techniques [32]. Briefly, samples were grown on Potato Dextrose Agar (PDA, Difco) at 27 °C for several days. Several cultures (5–10) of single fungi colonies of each isolate, which were obtained by micromanipulation, were cultivated and maintained in potato dextrose broth media (PDB, Difco) in different batches. These cultures were grown for 3–10 days under continuous shaking conditions and at a temperature of 25 °C.

Samples of the fungi were purified from the medium by centrifuging about 1.5 ml of the mixture at 16168*g* (13,200 rpm) for 4 min, washing the pellets 4 times with distilled water and suspending it in an appropriate volume of distilled water (about 1 ml) for spectroscopic measurements.

2.2.1. Pure Fungal Samples

The fungi were first cut and grounded into small pieces and then mixed very well with distilled water obtaining semi-homogenous suspension since it is impossible to dissolve mycelial pellets in water. About 1–2 µl of each fungal suspension sample was dropped on ZnSe crystal, air dried for about 30 min and measured by FTIR microscopy.

2.2.2. Mixed Fungal Samples

From each pure fungus, one and half milliliter was well mixed with the same amount of another fungus, in order to generate the homogeneous (as possible) mixed fungal sample used in this study. About 1–2 µl of the mixture was dropped on the ZnSe crystal for FTIR measurements.

2.3. FTIR Measurements

The detailed information of the investigated isolates is listed in Table 1. All measurements were performed using Thermo Scientific™ Nicolet™ Continuum™ Infrared Microscope with a liquid-nitrogen cooled MCT detector, coupled to the FTIR spectrometer "Nicolet iN10." in the transmission mode. Each spectrum is an average of 128 co-added scans with spectral resolution of 4 cm^{-1} in the mid-infrared 600–4000 cm^{-1} region. The background was measured first from each slide and then the samples were measured under the microscope to remove the influence of the environment.

2.4. Spectral Manipulation

The 2990–4000 cm^{-1} range is mainly due water absorption, while the 1775–2800 cm^{-1} range does not include any specific absorption band that is characteristic of the samples, so they were excluded from the measured spectrum. All the spectra contain two specific regions—(900–1775 cm^{-1} and 2800–2990 cm^{-1})—after spectral manipulation. Spectral manipulations were performed using OPUS 7 software and included baseline correction by the concave rubberband method; normalization was determined using the vector normalization method, and then offset-corrected.

2.5. Statistical Analysis

2.5.1. PCA

We used principal component analysis (PCA), which is a standard tool used in modern data analysis [33,34] or dimensional reduction. PCA is widely used in situations that feature identification problems. It is a mathematical algorithm for dimensionality reduction while preserving the direction, which contains the most variance (Hopefully, they are also the discriminative directions). In other words, instead of using many variables, the variability in the data is described by using fewer PCs [5].

Using PCA, we look for new directions (axes or loadings) in which the data are more separable. The loadings are arranged in a descending order according to their variance, thus PC1 has the largest variance. Looking on the data in this new domain, when the spectra are presented as a linear combination of the first few PCs, usually there is a good

Table 1
Details of the fungi samples used in this study for the two biological systems.

Pure samples	No. of measured pure samples	Mixture samples	No. of measured mixed samples
Colletotrichum	230	*Colletotrichum-Verticillium*	76
Verticillium	306	*Colletotrichum-Fusarium*	85
Fusarium	265	*Colletotrichum-Rhizoctonia*	72
Rhizoctonia	253	*Verticillium-Fusarium*	85
		Verticillium-Rhizoctonia	92
		Fusarium-Rhizoctonia	78