Identification of fungal phyto-pathogens by Fourier-transform infrared (FTIR) microscopy

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Fungi are considered as serious pathogens to many plants and can cause a severe economic damage. Early detection and identification of these pathogens is very important and might be critical for their control. The available methods for identification of fungi are time consuming and not always very specific. Fourier-transform infrared (FTIR) microscopy is proved to be a reliable and sensitive method for detection of molecular changes in cells. In the present study we used FTIR microscopy as a sensitive and effective assay for the detection and discrimination between different genera of fungi. Our results showed significant spectral differences between the various examined fungal genera.

Keywords: FTIR microscopy, fungal detection, fungi, spectral characteristics

Introduction

Fungal pathogens cause serious damage to large number of crops with a significant negative feedback on economy. Early identification enables precise targeting a pathogen and enables the most effective treatment. Most commercially available identification systems for fungi are based on the physiological characteristics or on serological methods . Such identification systems are usually time consuming and not always very specific. Between the techniques offering possibilities for rapid analysis, molecular biology methods are considered the most rapid and sensitive methods for identification of pathogens, but they are not yet in large-scale use.

The detection and identification of microorganisms using spectroscopic techniques promises to be of a great value because of its sensitivity, rapidity,

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low expense and simplicity. This together with the large information already known about spectral peaks obtained from FTIR spectra of living cells (Diem *et al*., 1999), make FTIR spectroscopy as an attractive technique for detection and identification of pathogens. This technique was used for the detection and characterization of cancer cells (Rigas *et al*., 2000; Huleihel *et al*., 2002), cells infected with viruses (Salman *et al*., 2002) and microorganisms including fungi (Gordon *et al*., 1999; Mariey *et al*., 2001; Maquelin *et al*., 2003).

In the present study we used FTIR microscopy for the identification and discrimination between different genera of fungi, which are considered as sever pathogens for various crops.

Materials and Methods

Fungi

In the present study we used various fungi genera causing serious damage to different plants as follows:

1. *Pythium* spp. – the causal agent of Damping-off disease on vegetables and flowers.
2. *Fusarium* spp. – the causal agent of wilt diseases in various crops.

From each genus 20 different species were examined. All fungi were supplied by our co-author L. Tsror from the Department of Plant Pathology in Gilat Experiment Station, ARO, Israel. These fungi were grown on Potato Dextrose Agar (PDA) (Difco) for several days in 27°C.

Sample preparation

Since ordinary glass slides exhibit strong absorption in the wavelength range of interest to us, we used zinc selenide crystals, which are highly transparent to IR radiation. Small aliquots of fungi were picked up from 3-days-old fungal colony with a bacteriological loop, suspended in 100 µl of saline, pelleted by centrifugation at 1000 rpm for 2 minutes. Each pellet was suspended with 20 µl of saline and a drop of 1 µl of the obtained suspension was placed in a certain area on the zinc selenide crystal, air dried for 15 minutes at room temperature (or for 5 minutes by air drying in a laminar flow) and examined by FTIR microscopy.

FTIR spectra measurement

FTIR measurements were performed in the transmission mode with a liquid-nitrogen-cooled MCT detector of the FTIR microscope (Bruker IRScope II) coupled to an FTIR spectrometer (BRUKER EQUINOX model 55/S, OPUS software). The spectra were obtained in the wave number range of 600-800 cm^{-1}. Spectral resolution was set at 4 cm^{-1}. Baseline correction by the rubber band method and vector normalization were obtained for all the spectra by OPUS software. Peak positions were determined by means of a second derivation method by OPUS software. Since the samples to be analysed were often heterogeneous, appropriate regions were chosen by FTIR microscopy so as to eliminate different impurities (salts, medium residuals, etc.). The aperture used in this study was 100 micrometers, since this aperture gave the best signal/noise ratio. At lower apertures, the quality of the spectra was bad due to the high noise level. For each sample, the spectrum was taken as the average of five different measurements at various sites of the sample. Each experiment with each sample was repeated five times. It is important to mention that there were no significant differences in the spectra from various sites (SD did not exceed 0.005).

Results and discussion

FTIR spectra of fungi

Samples obtained from two different fungal genera were examined by FTIR microscopy trying to find specific spectroscopic biomarkers for rapid identification and discrimination between fungal genera. Developing specific biomarkers by FTIR microscopy could be highly important for future rapid and reliable detection and identification of these pathogens.10 species from each fungal genera were examined.

Our results presented in Fig.1 show the average FTIR spectra of the tested species of *Pythium* spp. and *Fusarium* spp. It can be seen that despite the general similarity between the spectra of these different genera, there is a unique spectrum for each one with specific differences compared to the other genus. These results provide a preliminary indication of possible spectral parameters for identification of fungal genera. These results are in agreement with previously published results showed spectral unique "finger print" for each of large number of bacterial strains (Maquelin *et al*., 2003).

For all examined fungal spectra, the dominant bands at 1655 cm^{-1} and 1546 cm^{-1} were attributed to protein amide I and II bands (Dukor, 2002). The

shoulder at about 1750 cm^{-1} was attributed to lipid C = O stretching vibrations (Dukor, 2002). The band at 1465 cm^{-1} was assigned to the CH_2 bending mode

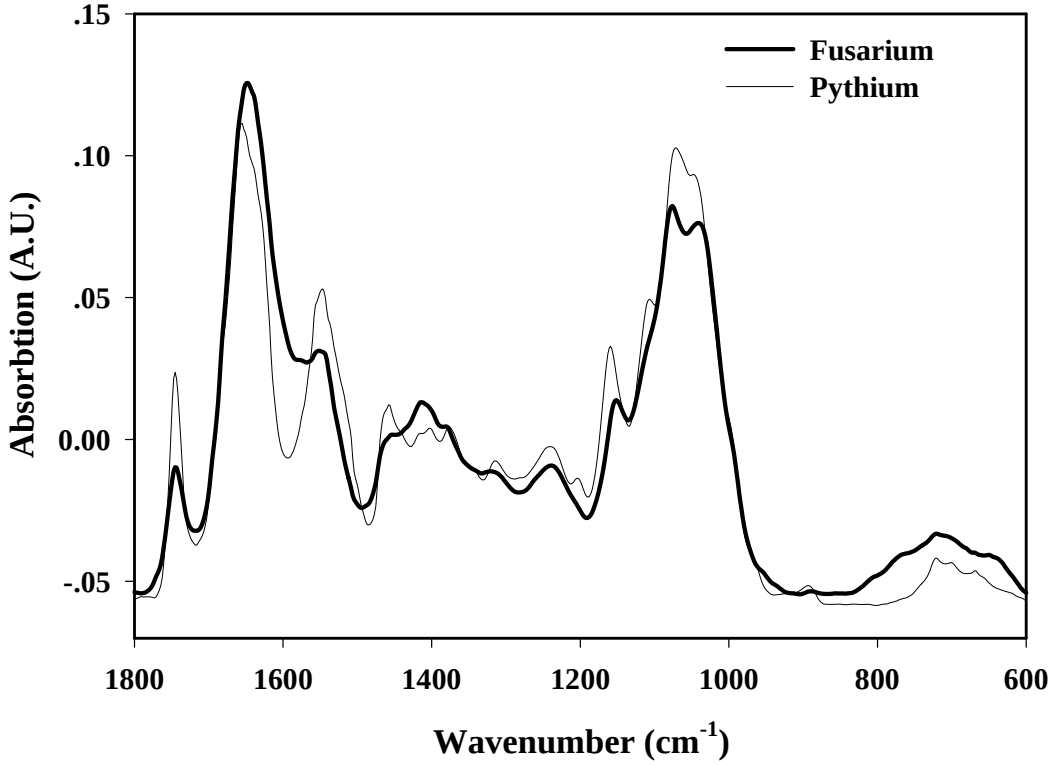

Fig. 1. FTIR spectra at the region 600-1800 cm^{-1} of the different examined fungal genera (*Pythium* spp.; *Fusarium* spp.). Results are means of 20 different species of each genus and separate experiments for each sample. The SD for these means was ≤ 0. 01.

of the cell lipids. The band at 1460 cm^{-1} represents asymmetric CH_3 bending modes of end ethyl groups of proteins (Wong *et al*., 1993). The band at 1402 cm^{-1} represents C = O symmetric stretching of COO^- (Maquelin *et al*., 2002) and assigned to lipids (Wong *et al*., 1993) and the band at 1377 cm^{-1} represents C-H bending mode of CH_2 (Brandenburg and Seydel, 2002). From information obtained from previous studies (Dukor, 2001) we assigned the remaining IR bands as follows: The peaks at 1237 cm^{-1} and 1082 cm^{-1} were attributed to PO_2^- asymmetric and symmetric stretching vibrations and phospholipids. The peak at 1064 cm^{-1} resulted from the overlap of several bands, including absorption due to the vibration modes of CH_2OH and the C–O stretching

vibration coupled to the C-O bending mode of cell carbohydrates (Yang *et al*., 1995).

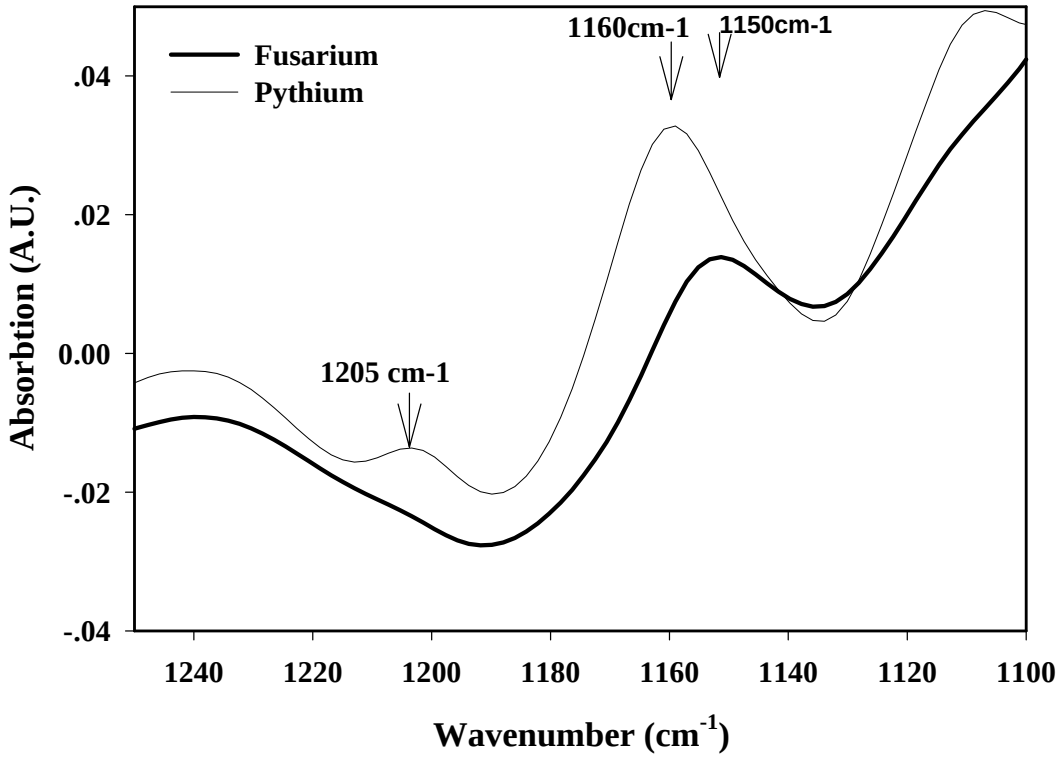

Fig. 2. FTIR spectra at the region 1100-1250 cm^{-1} of *Pythium* spp. and *Fusarium* spp.

Discrimination between the examined fungi genera

1. A peak at the 1160 cm^{-1} region appeared *si*gnificantly in all examined species of the genus *Pythium* (Fig. 2). This peak appeared at 1150 cm^{-1} in all tested species of the genus *Fusarium*. The peak at this region represents C=O stretch.

2. A peak at 1205 cm^{-1} region unique to the genus *Pythium*. Fig. 2 also shows a significant and detectable peak at 1205 cm^{-1} (which assigned to C-C-N

bending) in all tested species of *Pythium*. This peak is missing in all examined species of *Fusarium*. This peak represents CH_3 symmetric deformation.

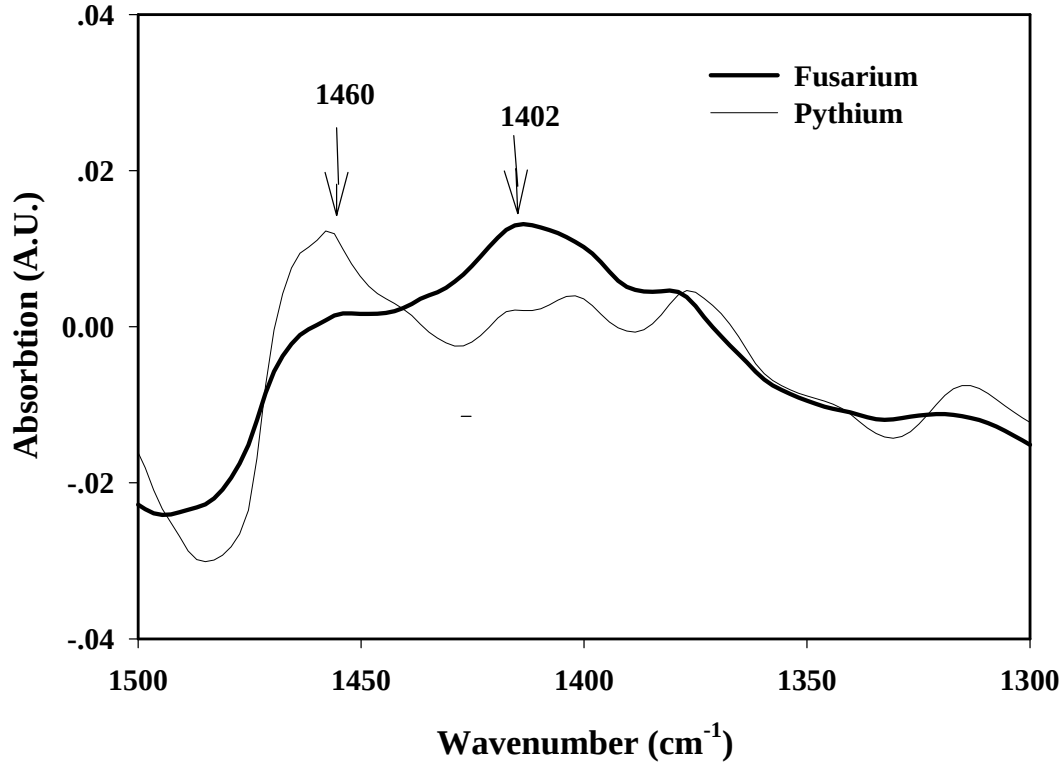

Fig. 3. FTIR spectra at the region 1300-1500 cm^{-1} of *Pythium* spp. and *Fusarium* spp.

3. It was found that peak ratio 1460 cm^{-1}/ 1402 cm^{-1} is consistently lower than 1 in all examined species of *Fusarium* and higher than 1 in all tested species of *Pythium* (Fig. 3). As mentioned above, the band at 1402 cm^{-1} represents C=O symmetric stretching of COO^- and assigned to lipids and the band at 1460 cm^{-1} represents asymmetric CH_3 bending modes of end ethyl groups of proteins.

Conclusions

In the present study we examined the potential of FTIR microscopy for an easy and rapid discrimination and identification of various fungi genera,

which are responsible for a serious damage to agriculture. The results obtained in this study:

1. Provide a unique and consistent spectral marker/s for each of the examined fungi genera.
2. Show that the spectral area ranged between 1000 to 1800 cm^{-1} can be considered as an important area for an easy and reliable discrimination between the various examined fungi genera.

Additionally, the fact that the final results could be obtained during very short time (approximately 1 hour) from a small amount of sample, support the possibility of developing FTIR spectroscopy as a reliable method for rapid identification of fungal infections in plants.

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