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PREPARATION PROCEDURES OF FUNGAL PHYTO-PATHOGENS SAMPLES FOR ANALYSIS BY MALDI-TOF AND FTIR MICROSCOPY

V. Erukhimovitch[1], L.Tsror (Lahkim)[2], M.Karpasas[1], M.Hazanovsky[2], M.Huleihil[3], M.Huleihel[4]

[1]*Analytical Equipment Unit, Ben-Gurion University of the Negev,Beer-Sheva, Israel.*
[2]*Department of Plant Pathology, The Institute of Plant Protection, Agricultural Research Organization, Gilat Experiment Station, M.P. Negev, 85250, Israel.*
[3]*Department of Physics, Sami Shamoon College of Engineering , Beer-Sheva 84100, Israel.*
[4]*Department of Virology & Developmental Genetics, Faculty of Health Sciences, Ben-Gurion University of the Negev,Beer-Sheva, Israel.*

Abstract

Reliable and rapid identification of phyto-pathogens causing plant diseases is playing an important role in their control strategies. The available methods for identification of fungi are time consuming and not always very specific. MALDI-TOF and Fourier-transform infrared (FTIR) microscopy are proved to be comprehensive and sensitive analytical methods for detection of molecular changes in cells. Due to the similarity between the obtained spectra of different species of fungal pathogens, it is important to choose the most appropriate procedure for the preparation of the examined samples. Such procedure might improve the discrimination between these species. In the present study, we compared between three possible procedures of pathogen sample preparation for their examination by MALDI-TOF and FTIR microscopy. Our results showed that preparation of the fungal sample directly from liquid growth media is considered as the best way of fungal sample preparation for both MALDI-TOF and FTIR microscopy examinations.

Keywords: *MALDI-TOF, FTIR microscopy, Fungi, Spectral characteristics, fungal detection, agar.*

1. INTRODUCTION

Fungal pathogens are considered as one of common causative of severe diseases in various plants leading, in many cases, to a large economic damage [1], for instance *Colletotrichum coccodes* – a major pathogen of potato and tomato [2]. Early identification enables to precisely target a pathogen with the most effective treatment saving a large economic damage. Most commercially available identification systems are based on the physiological and nutritional characteristics of fungi. Such identification systems are usually time consuming 2-4 weeks and

not always very specific. The detection and identification of microorganisms by spectroscopic techniques promises to be of a great value because of their sensitivity, rapidity, low expenses and simplicity. Furthermore, spectroscopic techniques provide a wealth of qualitative and quantitative information about a given sample. The spectrum of any compound is known to give a unique "finger print" [3, 4]. This together with the large information already known about spectral peaks obtained from MALDI-TOF and FTIR spectra of living cells [5,6], make these spectral techniques as an attractive for detection and identification of intact biomolecules and living cells including pathogens. These techniques were used previously for the detection and characterization of cancer cells [7, 8] cells infected with viruses [9,10] and microorganisms including some fungi [4,6,11, 12, 13]. In the present study, we compared between the reliability of three possible procedures for the preparation of fungal slides appropriate for examination by MALDI-TOF and FTIR microscopy.

2. EXPERIMENTAL

Fungi

In the present study we used two different isolates (112 and 136) of the fungi genus *Colletotrichum coccodes* – a major pathogen of potato and tomato. Fungi cultures were grown in 27 □ C for 3-4 days, either on solid medium - Potato Dextrose Agar (Difco), or in liquid culture, Czapek Dox Broth (Difco), with continuous shaking (500 rpm).

Procedures used for the purification of fungi samples:

Procedure 1. A small aliquots of fungi were picked up from the growing fungi on the agar with a bacteriological loop, suspended in 100µl of saline, pelleted by centrifugation at 1000 rpm for 2 min. Each pellet was suspended with 20µl of 0.1% TFA (for MALDI-TOF) or H_2O (for FTIR microscopy).

Procedure 2. A fungi samples were picked up from the growing fungi on the agar by adding 1ml of distilled water to the plate, shaking it gently and collecting the water containing suspended fungi to a new tube. The fungi were pelted from this suspension by spinning it at 2000 rpm for 5 min, washed twice with H_2O and the pellet was suspended in appropriate volume (about 50 µl) of 0.1% TFA or H_2O.

Procedure 3. The fungi were grown in appropriate liquid media. Samples of these fungi were purified from these media by spinning about 1ml of medium containing fungi at 2000 rpm for 5 min, washing twice with H_2O and the pellet was suspended in appropriate volume (about 50 µl) of 0.1% TFA or H_2O.

Sample preparation for MALDI-TOF

Fungal samples dissolved in 0.1% TFA were dialyzed for 30min by spotting on a 0.25µm MF-Millipore membrane filter (Millipore, Bedford, MA, USA) floating on distilled water. After dialysis the samples were removed and mixed with an equal amount of saturated sinapinic acid solution. Sinapinic acid (Aldrich, Milwaukee, W1, USA) was dissolved in water-acetonitrile (1:1) until saturation occurred. Samples were spotted on an autosampler plate and allowed to air dry.

MALDI-TOF analysis

Samples were analyzed on a Reflex IV (Bruker Daltamics, Germany) MALDI-TOF instrument using 337nm radiation from a nitrogen laser. An accelerating voltage of 20kv was used. The spectra were recorded in linear mode within a mass range from m/z 2000 to 9,000.

Sample preparation for FTIR microscopy.

A drop of 1µl of the obtained fungi suspension was placed on a certain area on zinc sellenide crystal, air dried for 15min at room temperature (or for 5 min 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 600-1800 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 analyzed were often heterogeneous, appropriate regions were chosen by FTIR microscopy so as to eliminate different impurities (salts, medium residuals, etc.). 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 and average of obtained results was determined.

3. RESULTS AND DISCUSSION

*MALDI-TOF spectra of fungi strains 112 and 136 prepared by different preparation procedures*Fungi isolates 112 and 136 of *Colletotrichum coccodes* genus were prepared by three different procedures (as detailed in Materials and Methods section) and examined by MALDI-TOF. The results presented in Figs. 1 and 2 show representative spectra of these fungi obtained by each of the used purification procedures at the region m/z 2,500-9,000. It can be seen clearly that samples obtained by procedure 3 (Figs.1C and 2C) show best spectral peaks compared to

procedures 1 (Figs.1A and 2A) and 2 (Figs.1B and 2B).Worst results were obtained with procedure 1, probably due to contamination with agar leftovers which may affect MALDI-TOF performance. In addition, the presented results show spectral peaks specific to each of the examined fungal strains with significant differences between both of them. These peaks may be used in the future as biomarkers for easy and rapid discrimination between these strains.

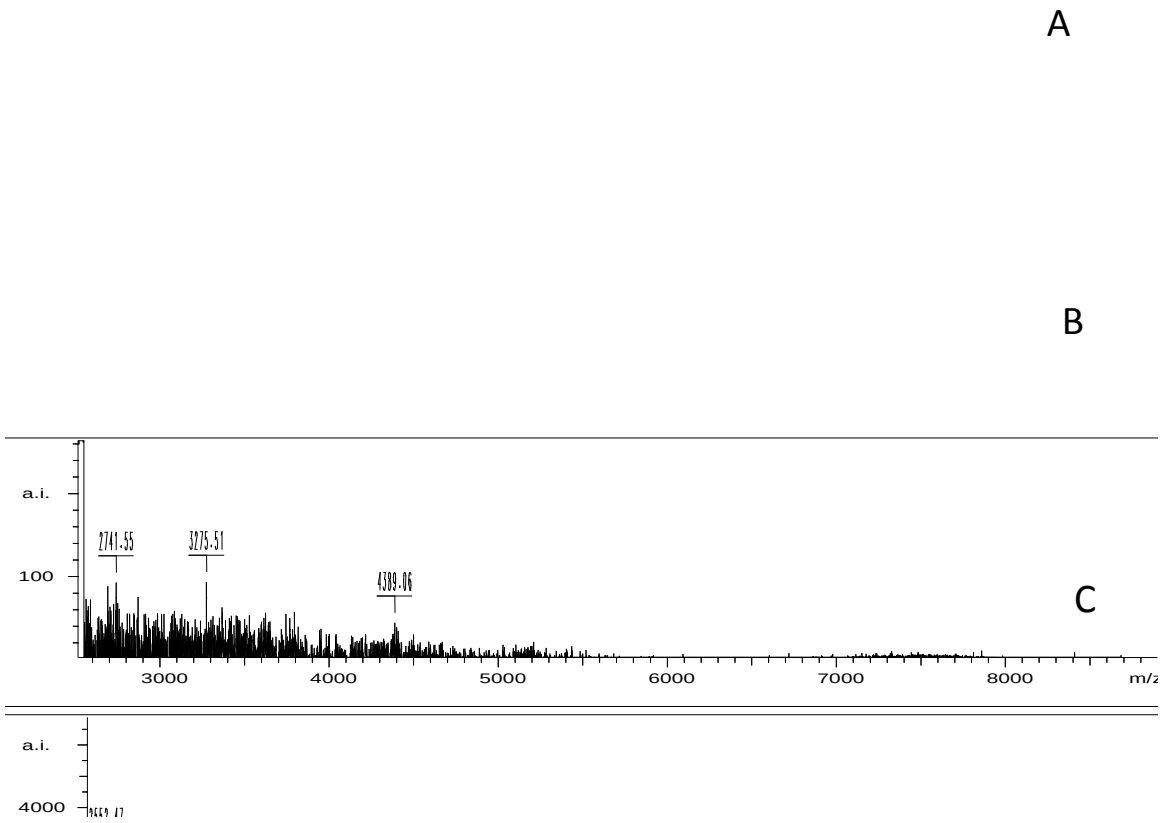

Figure 1 MALDI-TOF spectra in the region 2000 to m/z 9,000 of fungi isolate 112 prepared and purified by (A) procedure 1 (B) procedure 2 and (C) procedure 3.

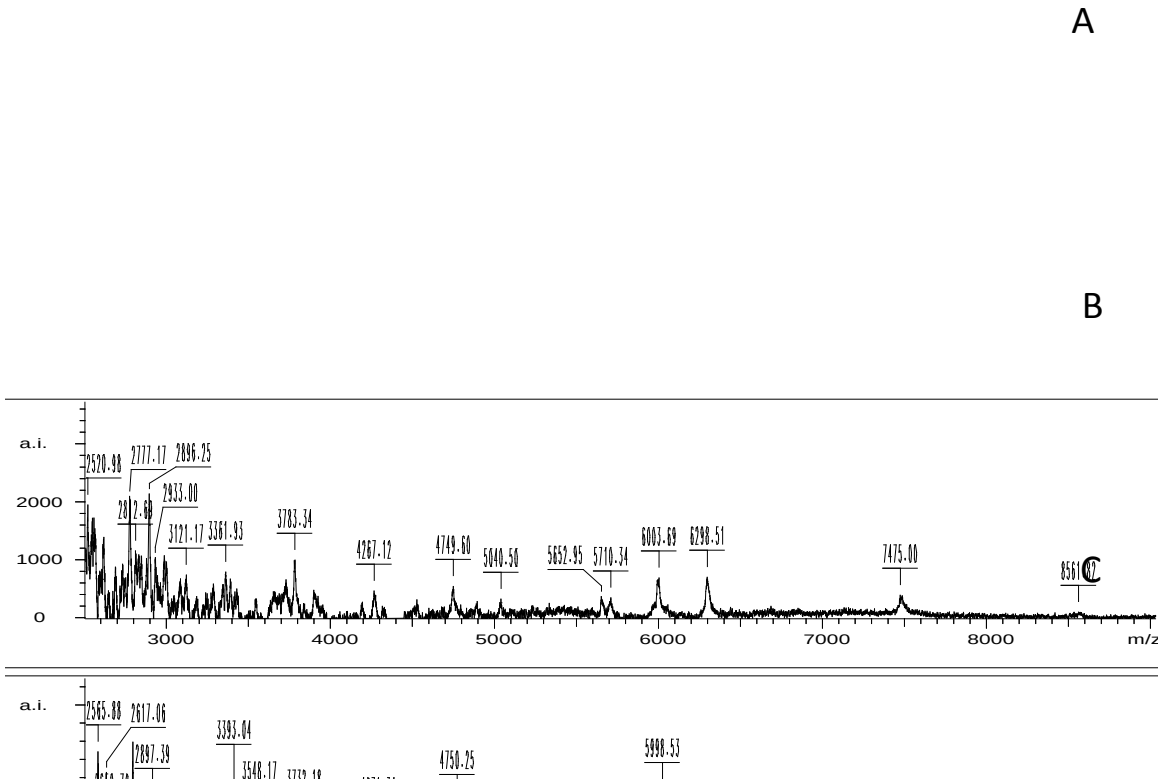

Figure 2 MALDI-TOF spectra in the region 2000 to m/z 9,000 of fungi isolate 136 prepared and purified by (A) procedure 1 (B) procedure 2 and (C) procedure 3.

FTIR spectra of fungi strains prepared by the different preparation procedures

Fungi strains 112 and 136 were prepared by the above-mentioned procedures and examined by FTIR microscopy. The results presented in Figs.3A and 4A show the average FTIR spectra of fungi specie 112 and 136 respectively obtained by either of the examined procedures. It can be seen that despite the general similarity between the obtained spectra, there are specific differences between the different used procedures. It seems that these spectral results might be affected by agar leftovers due to very strong absorbance peaks of a pure agar at the area 1000-1100 cm^{-1} which represents carbohydrates bands (Figs. 3A, 4A). These significant absorbance peaks of the agar can strongly affect the spectra of the fungi if the obtained fungi samples are not completely purified from all agar leftovers.

Comparison of IR spectra in the1000-1100 cm^{-1} region

As can be seen from Figs. 3B there are very strong absorbance peaks of the agar at 1044 and 1075cm^{-1}. The spectrum of the examined fungi obtained by procedure 1 at this region show two clear spectral peaks located approximately at the same position of the peaks obtained

from pure agar as can be seen in Figs. 3B, 4B. In contrast, the results obtained by procedure 2 showed also two peaks at this region but with a shift compared to the agar peaks and peaks obtained in procedure 1 (Figs. 3B, 4B)**.** Procedure 3 showed two peaks at this region with an impressive shift compared to the agar peaks (Fig.3B, 4B).

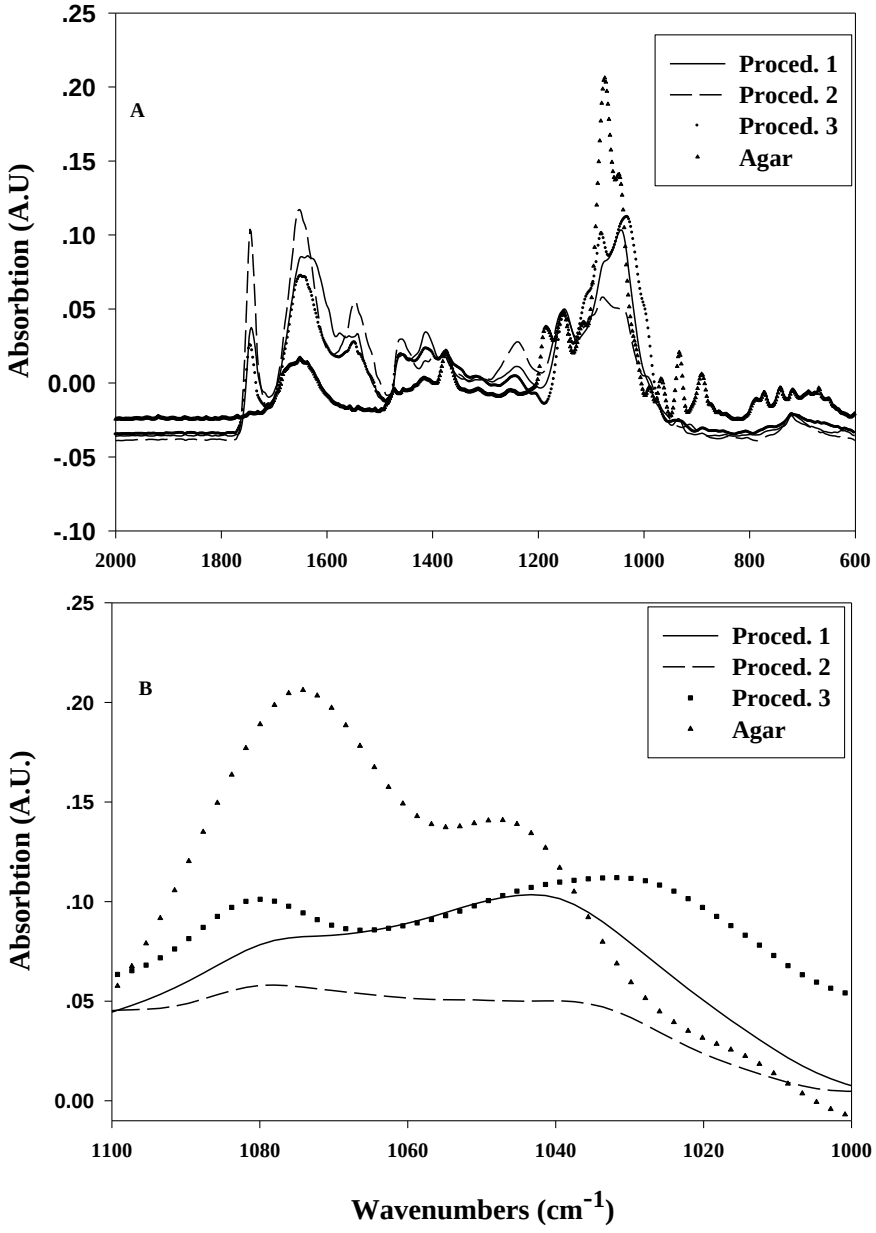

Figure 3 FTIR spectra of fungi isolate 112 prepared by three different procedures and of pure agar. (A) at the region 600-2000 cm^{-1} and (B) at the region 1000-1100 cm^{-1}. Results are means of 5 different members of this species and separate experiments for each sample. The SD for these means was ≤ 0. 01

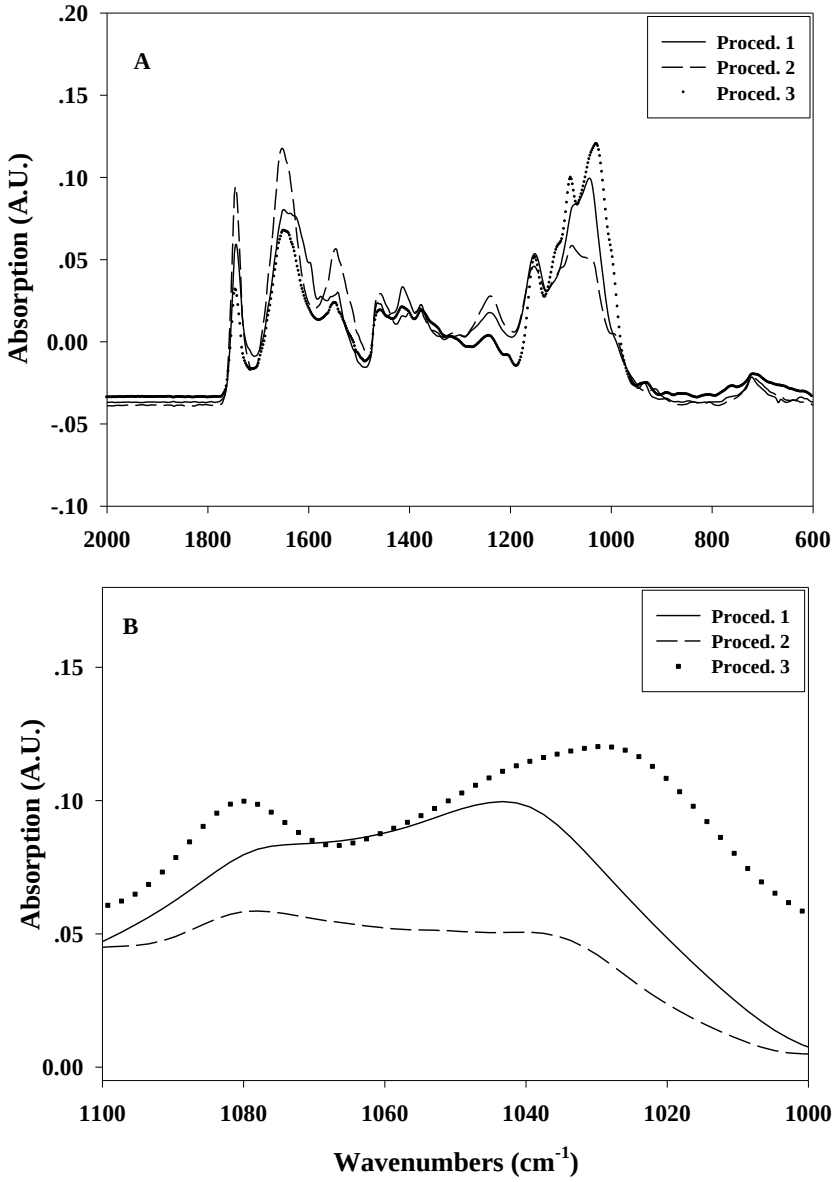

Figure 4. FTIR spectra of fungi isolate 136 prepared by three different procedures. (A) at the region 600-2000 cm^{-1} and (B) at the region 1000-1100 cm^{-1}. Results are means of 5 different members of this species and separate experiments for each sample. The SD for these means was ≤ 0.01.

Plants are threatened by a numerous number of fungal pathogens, which belongs to different genera. Fungal infections can cause a serious damage to the crop, directly affecting economy. In each fungal infection, early identification of the fungal pathogen plays a critical role in choosing the most effective way of treatment. Spectroscopy is taking a more and more important place in discrimination and identification of microorganisms [11]. Problems of reproducibility that faced scientists [14], seem to be resolved, providing standardized conditions for cell growth and

sample preparation [11]. Along with these precautions, different scientists succeeded to improve dramatically their ability to identify various microorganisms by spectroscopic methods [12, 15-17].

In the present study, we evaluate MALDI-TOF and FTIR microscopy potential for identification of fungal pathogens. As a first step, we examined and evaluated several methods of the fungal slides preparation, choosing the most reliable one for our coming analysis. Our results showed that procedure 1, which based on picking up the fungi directly from the agar, is the worst and cannot be used for identification of fungi due to a serious contamination with agar leftovers. The FTIR microscopy results (Figs. 3, 4) show that pure agar has very strong spectral peaks at 1000-1100 cm^{-1} region which completely shield on the real spectral peaks of the fungi at this region and also may interfere with the performance of MALDI-TOF examination. Whereas, procedure 3, based on growing the fungi in liquid media without agar, provided the best results without any effect of agar. Procedures 2, based on suspending the fungi in distilled water from the agar provided also satisfied results.

4. CONCLUSIONS

Our results showed that procedure 3, based on growing the fungi in liquid media without agar, provided the best results for identification of fungi without any effect of agar.

Procedure 1, which based on picking up the fungi directly from the agar, is the worst and cannot be used for identification of fungi due to a serious contamination with agar leftovers.

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