Identifying and detecting Entomopathogenic fungi using Surface-enhanced Raman spectroscopy

Identificação e detecção de fungos entomopatogênicos utilizando Superfície-enhanced Raman espalhamento

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ABSTRACT
In the natural ecosystem, fungal entomopathogens are the most efficient biocontrol agents against insect pests. In this study we offer an alternative for conventional fungal diagnostic, Surface-enhanced Raman spectroscopy (SERS) technique combine with principal component analysis (PCA) for detection and identification three entomopathogenic fungi, namely, IBCB 66 Beauveria bassiana, IBCB 130 Isaria fumosorosea, and IBCB 425 Metarhizium anisopliae. Using a simple preparation approach, highly active silver nanoparticles suitable for detecting complex biomolecules were produced for application in the SERS technique. Entomopathogens fungi produced highly enhanced and reproducible Raman signals based on their biochemical composition due to the high density of hot spots at the confluence of silver nano-aggregates, allowing the three entomopathogens species to be differentiated in the SERS spectrum fingerprint region, 550-1700 cm\(^{-1}\). The SERS method, along with PCA analysis, accounted for over 99 % of total variance and allowed for very high probability discrimination between the three entomopathogens, allowing taxonomic affiliation to be determined in a short period of time. These findings suggest that the SERS methodology can be used to develop a new, fast, accurate, and cost-effective diagnostic method for fungal entomopathogens.

Keywords: SERS, entomopathogenic fungi, Metarhizium anisopliae, Beauveria bassiana, Isaria fumosorosea, detection.
RESUMO

No ecossistema natural, os fungos entomopatogênicos são os agentes de biocontrole mais eficientes contra as pragas de insetos. Neste estudo, oferecemos uma alternativa para o diagnóstico fúngico convencional, a técnica de Superfície-enhanced Raman espalhamento (SERS) combinada com a análise de componentes principais (PCA) para detecção e identificação de três fungos entomopatogênicos, a saber, IBCB 66 Beauveria bassiana, IBCB 130 Isaria fumosorosea, e IBCB 425 Metarhizium anisopliae. Usando uma abordagem de preparação simples, nanopartículas de prata altamente ativas adequadas para a detecção de biomoléculas complexas foram produzidas para aplicação na técnica SERS. Fungos entomopatogênicos produziram sinais Raman altamente aprimorados e reproduzíveis com base em sua composição bioquímica devido à alta densidade de pontos quentes na confluência de nanoagregados de prata, permitindo que as três espécies de fungos sejam diferenciadas na região do espectro SERS, impressão digital, 550-1700 cm⁻¹. O método SERS, junto com a análise de PCA, foi responsável por mais de 99% da variância total e permitiu uma discriminação de probabilidade muito alta entre os três fungos, permitindo que a afiliação taxonômica fosse determinada em um curto período de tempo. Esses achados sugerem que a metodologia SERS pode ser usada para desenvolver um método de diagnóstico novo, rápido, preciso e de baixo custo para fungos entomopatogênicos.

Palavras-chave: SERS, fungos entomopatogênicos, Metarhizium anisopliae, Beauveria bassiana, Isaria fumosorosea, detecção.

1 INTRODUCTION

Entomopathogenic fungi (EPF) are worldwide natural enemies of arthropod pests that are effective in controlling a variety of insect pests in natural habitats while being environmentally friendly. The IBCB 66 Beauveria bassiana and IBCB 130 Isaria fumosorosea but IBCB 425, and Paecilomyces are predominant genera of EPF widely used as biocontrol agents throughout the world. The white muscardine fungus B. bassiana and the green muscardine fungus M. anisopliae, are the most well-known fungal entomopathogens for the control of sucking and chewing agricultural insect pests and play an important role in the integrated pest management systems (Malekan et al., 2015). The B. bassiana is reported to infect 707 species of insect hosts (Imoulan et al., 2016) whereas M. anisoplae is infecting over 200 species of insect pests (Jitendra et al., 2012). The B. bassiana (Balsamo) Vuillemin (Ascomycota: Hypocreales) has been exploited extensively to control insects that affect crops or are vectors of human and animal diseases. Aside from its entomopathogenic lifestyle, several B. bassiana species are able to grow endophytically inside the plants and offer protection against pests and pathogens of different host plants, defining its role in agricultural food production systems. Various studies have also shown that native EPF isolates are efficient against a variety of agricultural pests in local settings (Clifton et al., 2019). The diamondback moth Plutella xylostella is related as one of many pest in cruciferous worldwide. It has been reported that B. bassiana, I. fumosorosea and M. anisoplae have successfully biocontrolled Plutella xylostella larvae and pathogenicity on eggs, larvae, pupae and adults of the pest (de
Almeida, 2009). The horn fly *Haematobia irritans*, is a major bovine external parasite in the USA and around the world, causing millions of dollar en losses each year (Li et al., 2007). The efficacy of EPF in the management of *H. irritans* eggs and larvae was documented in studies exploring the potential of EPF in the control of *H. irritans*, notably employing *I. fumosorosea* (Alves et al., 2010). Coffee Berry Borer (CBB) Microbial Control with *B. bassiana* is the most researched fungus for coffee pest control, with the majority of research focusing on CBB control. It's been identified as a naturally occurring adult CBB pathogen all over the world (Alves, 1998; Alves et al., 2008; Gallo et al., 2002; Rocha L., 2008. On the other hand, the inundative application of *B. bassiana* for CBB control can be timed to reduce the pest population effectively based on the time of berry invasion and appropriate environmental circumstances. The elimination methods begin with the conidia or spores of these EPF germinating on the surface of their insect host, penetrating through the cuticle, spreading systemically in the hemolymph, and finally causing the host's death; consequently, entomopathogenic fungus tend to kill the animal slowly. These crop diseases, on the other hand, are typically managed through the use of chemical insecticides. However, because of the indiscriminate application of high-concentration of chemicals, the parasite develops resistance, reducing effectiveness of these treatments (Oremus et al., 2006). Aside from the expected poisonous effect, chemical substances have the potential to harm other living species as well as the environment (Mochi et al., 2005). Furthermore, these treatments can help to limit the usage of chemicals and aid to environmental preservation (Angel-Sahagun et al., 2005; Lohmeyer and Milller, 2006). These research revealed that employing EPF isolates to control insect pests is more successful and may be a promising option in terms of environmental appropriateness with pest species (Imoulan et al., 2011; Lee et al., 2015). Isolation, identification, and screening of indigenous EPF as bio-control agents is essential for identifying novel virulent isolates for successful insect pest management in greenhouse and field environments. Traditional methods of fungal identification rely on the cultivation of sampled organisms as well as careful examination of their macro- and microscopic morphological characteristics. DNA/RNA extraction procedures, polymerase chain reaction (PCR) amplification, and DNA-denaturing gradient gel electrophoresis (DGGE) are not bias-free, and each step can influence the results in the end. They also have several flaws, such as polymerase errors, size limitations, and/or non-specific priming and profiles. bioinformatic analysis parameter settings. Differences in DNA extraction processes, sequencing technology, and platform usage result in data sets that have little, if any, in common for statistical comparison (Naja, et al., 2007, Rabinow, 1996). In addition, molecular technologies is relatively expensive, labor and time consuming, and destructive in nature, requiring considerable sample preparation as well as highly-trained expert individuals for
analysis (Ramirez-Perez, 2013, 2019). Raman spectroscopy, which is based on vibrational transitions in molecules, looks to be more adapted to studying biological systems, such as Raman scattering (Stöckel et al., 2015). In biological systems, where several of the target biocompounds are present at low concentrations, normal Raman (NR) spectroscopy is rather unselective with respect to the numerous molecules present in the biological milieu, given very congested spectra. In addition, in NR, there is frequently a significant fluorescence background (intrinsic or impurity-derived) that degrades the spectral quality and reduces the signal-to-noise ratio (Efrima & Zeiri, 2008). However, bringing molecules and molecular structures close to noble metal surfaces such as Au and Ag nanoparticles might improve NR spectroscopy (NPs). Because of the significant increase it gives with visible excitation wavelengths, silver is the most commonly used metal, and aggregates are the ideal substrates for analytical purposes. Surface-enhanced SERS (single-emission Raman scattering) is an emerging technology for detecting, identifying, and characterizing chemicals and structures, such as microorganisms (Karaman et al., 2008). SERS is also a promising technology for biomedical applications in whole-organism fingerprinting research. The amplification of Raman signal from molecules in close proximity to a nanostructured surface is observed in the SERS technique due to the connection of metal surface plasmons with the oscillating electric field of incident and scattered radiation. The capacity of SERS-active substrates to quench fluorescence also increases the signal to noise ratio dramatically. The Raman signal is enhanced by two mechanisms: the first is an electromagnetic enhancement mechanism, and the second is a chemical enhancement mechanism. Light is amplified by activation of specific surface plasmon resonances, resulting in electromagnetic enhancement (LSPRs). This light is concentrated mostly in the nanogaps or sharp nanostructures of plasmonic materials like AgNPs. Reproducible and robust structures that strongly enhance the electromagnetic field are most desirable for SERS. Electromagnetic enhancement for SERS might be possibly reach factors of approximately $10^{10}$ depending on the structure of the supporting plasmonic material. In most circumstances, the enhancement factor can be well approximated by the magnitude of the localized electromagnetic field raised to $10^4$. Chemical enhancement, on the other hand, uses charge transfer pathways in which the excitation wavelength is resonant with the metal-molecule charge transfer electronic states. SERS research continues to focus on the development of substrates with high enhancement factors. The ability of SERS-based metallic NPs to detect and identify a variety of microorganisms has been demonstrated (Chen et al., 2015; Yung et al., 2016), spores (Cowcher et al., 2013) or fungi (Pan, T-t et al., 2017). Since SERS sample preparation consists of simply mixing a microorganism solution with aggregate NPs and then detecting the combination in a cuvette or drying the mixture on a glass slide for Raman spectrum analysis. In this paper, we show how the SERS technique may
be used to identify and detect EPF in a label-free and time-efficient manner. To our knowledge no SERS investigations have been conducted on EPF. In order to apply the SERS technique, we set up a sample preparation protocol, fabricated and analyzed AgNPs. The SERS signals of three of the most common EPFs were measured and in combination with PCA we performed the statistical analyses for detection and identification three EPF studied.

2 MATERIALS AND METHODS

2.1 ENTOMOPATHOGENIC FUNGI STRAINS

Fungi used in the study were IBCB 425 *Metarhizium anisopliae*, IBCB 66 *Beauveria bassiana* and, IBCB 130 *Isaria fumosorosea*. These EPF were obtained from entomopathogenic culture collection “Odemar Cardim de Abreu” of Biological control laboratory at Institute Biologico, Sao Paulo, Brazil. These strains were stored at -80oC stocked, dissolved and inoculated in sterile slant test tubes (100 mL) containing a potato-dextrose-agar (PDA) medium PDA and incubated at 25oC for 5 to 7 days in the dark, and after that held for 4-5 weeks at 4oC. The entomopathogenic fungi selected were cultured in 90 mm diameter Petri dish containing PDA, each plate was incubated at 25oC for 5 days in the dark. A homogenous sample (conidia and hyphae) from a colony was collected by scraping with a sterile scalpel and suspending in 500 µL distilled water (DI) in a 1.5 mL microtube, avoiding contamination by nutrients from culture media. The suspension was vortexed, rinsed, and centrifuged (12000 rpm/5 min); following centrifugation, the pellet was washed and re-suspended in a new 500 µL DI water, and the process was repeated three times. After vortexed and washed three times by centrifugation at 12000 rpm for 5 min, clean cells of fungi were suspended and kept in a 100 in µL eppendorf tube at 4oC. In this method, the sample, extraction, collection and preparation of clean fungal cells in form of aqueous solution from the NB was straightforward and quick. For SERS testing a 5 µL aliquot of AgNPs of different sizes was spotted onto a CaF$_2$ glass slide, followed by a 5 µL solution of clean fungal cells deposited onto each substrate, and the mixture was homogenized with a 10 µL pipette to serve as control, a 5 µL of clean fungal cells was spotted in the same CaF$_2$ glass slide. After forming a dried thin film, the samples were dried for about 10 minutes at room temperature before NR and SERS analysis. The experimental approach for the four fungi species investigated is depicted in Fig.1.

2.2 PREPARATION OF SILVER NANOPARTICLES

Silver nanoparticles (AgNP) were synthesized using glucose as reducing agent, and poly(N-vinylpyrrolidone) (PVP) was used as capping agent (Wang et al. 2005). The method is described
elsewhere (Banerjee et al., 2014). Briefly, in 320 mL, 16 g of glucose and 8 g PVP were dissolved in water and heated to 90 °C. Then immediately

Figure 1. Diagram of SERS technique. Cultures: A) IBCB 66 B. bassiana; B) IBCB 130 I. fumosorosea; C) IBCB 425 M. anisopliae; D) fungi culture preparation in PDA; E) Sample extraction; F) Sample preparation: G) Mix clean EPF cells with aggregates of AgNPs (60 nm); H) Cast mix sample onto CaF2 glass slide; I) EPF-AgNPs dried thin film active substrate; J) Raman spectroscopy analysis, 1% (500 mW) 785 nm excitation, 10 s exposure time to acquire SERS spectra.

dissolve 4 g AgNO3 in 8 mL of the mixture. The temperature of dispersion was maintained at 90 °C. At different intervals during the process, aliquots were collected and chilled to room temperature. Figure 1S (Supporting Information) displays the aliquot collected under reaction times 120 min (60 nm) minutes. The aggregates of AgNPs average diameter size was evaluated using Small-angle X-ray scattering (SAXS). The experimental scattering intensity data were described by a polydisperse sphere model (Linder, 1991 and Olivera et al., 2014):

\[ I(q) = Sc \left\{ \int_0^\infty V^2(R)D(R,\sigma)p_{sph}(q, R) dR \right\} S_G(q, RG) + B \]

where: R is sphere radius, \( \sigma \) is the standard deviation of the radius distribution (polydispersity), Sc is the scale factor, V(R) is the sphere volume, D(R,\( \sigma \)) is the radius distribution function (Schulz Zimm), \( p_{sph}(q, R) \) is the sphere form factor, q scattering vector of the reciprocal space, \( S_G(q, RG) \) is the Guinier structure factor, RG is the gyration radius of Ag nanoaggregate and B is the background. The Guinier factor describes the scattering of clusters with gyration radius equal to RG (Olivera et al., 2014). Figure 2S shows a typical UV-vis absorption band at 390 nm for 60 nm aggregates of AgNPs aliquot solution in the UV-vis spectrum.
2.3 RAMAN SPECTROSCOPY

The SERS measurements were performed using a Renishaw Raman microscope inVia Reflex Raman Microscopy System Leica DM2500 M (Ranishaw PlC., New Mills, Wotton-under-Edge Gloucestershire, UK) equipped with 500 mW (maximum high power) diode laser emitting a 785 nm line which was used as an excitation source. The laser light was passed through a line filter and focused on a sample mounted on a tridimensional stage with 50x objective lens (numerical aperture 0.75) that focused the laser to a spot size around 2.5 um. The Raman scattering signals were recorded by using a 1040x256 pixel RenCam CCD array detector with thermoelectrically cooling, 1200 lines network of diffraction was employed. The instrument was calibrated using a silicon wafer with the band center at 520 cm\(^{-1}\). During the measurement, just 1% of the nominal maximum high power light from the 785 nm diode laser was employed focused onto the sample at a microscope stage through a 50x objective with 10s exposure time, and 10 accumulations.

2.4 DATA PROCESSING

THE ORIGINPRO 8.6 SOFTWARE WAS USED TO TREAT THE NR AND SERS SPECTRA IN THIS INVESTIGATION. The AgNPs SERS active substrate was calibrated using pMBA typically standard SERS analyte, because it tends to adsorb efficiently on AgNPs surface (Le Ru et al., 2007). The SERS active-substrate exhibits high sensitivity, an enhancement factor approximately 4.4x10\(^7\), reproducibility (RSD=4%) and stability of near 2 months of the recorded SERS spectra (Figs. 3S and 4S, Supporting Information). The NR and SERS spectra for EPF studied were tested 3 times on different spots within the same sample. The experiments were repeated using the same experimental protocol (Fig.1) in three sessions during the period of April 30, June 05 and August 06, 2020.

3 RESULTS AND DISCUSSIONS

3.1 DETECTION AND IDENTIFICATION OF EPF STRAINS USING THE SERS TECHNIQUE

NR and SERS spectra of three EPF studied were recorded within a detection range from 550 to 1700 cm\(^{-1}\). There are no signals in the NR spectra of a clean CaF\(_2\) glass slide except for a broad weak band intensity with a maximum ca. 1400 cm\(^{-1}\) (Fig. 2a-4a). Due to sample background fluorescence, the NR of B. bassiana, I. fumosorosea, and M. anisopliae shows no discernible signals in this area except for a modest broad band at approx. 1400 cm\(^{-1}\) (Fig. 2b-4b). Three average SERS spectra of B. bassiana mixed with 60 nm AgNPs, on the other hand, show robust signals in the 1000 to 1700 cm\(^{-1}\) wavenumber range, as well as medium weak peaks outside of this wavenumber range (Fig.2c). The highest band intensity in the SERS spectrum of B. bassiana takes place at 1474 cm\(^{-1}\) (I\(_{1474}\) cm\(^{-1}\)), this enhancement might be attributed to the density of electromagnetic field between
aggregates of AgNPs and implying that the active substrate *B. bassiana*-AgNPs with smaller intraparticle gaps between neighboring AgNPs improves the active surface and facilitates contact with as many as biomolecules as possible of *B. bassiana*, resulting in a strong SERS signal enhancement near to $2.5 \times 10^4$. Similar behavior has been observed in the SERS spectrum of bacteria and many other biomolecules, suggesting an increase of adsorption of *B. bassiana* cells and their biomolecules components onto the AgNPs surface (Dina *et al*., 2017; Matsumoto *et al*., 2005; Pan, T-t. *et al*., 2017). Three average SERS spectra of *I. fumosorosea* (Fig. 3c) on average exhibit strong peak intensities in the region of 900-1600 cm$^{-1}$. The robust peak intensity ($I_{1254}$ cm$^{-1}$) achieved an enhancement ca. $7.0 \times 10^4$, which was much higher than *B. bassiana*’s enhancement for the selected peak at 1474 cm$^{-1}$. On the other hand, the average of three test SERS signals of *M. anisopliae* can be observed across the entire

Figure 2. SERS and NR Raman spectra of *B. bassiana*: (a) NR spectra of clean CaF$_2$ glass slide; (b) NR spectra (control); (c) 3 average SERS spectra. All spectra have been baseline corrected, and shifted vertically for improved visualization.

![Figure 2](image1)

Figure 3. SERS and NR Raman spectra of *I. fumosorosea* (a) NR spectra of clean CaF$_2$ glass slide; (b) NR spectra (control); (c) 3 average SERS spectra. All spectra have been baseline corrected, and shifted vertically for improved visualization.

![Figure 3](image2)
Figure 4. SERS and NR Raman spectra of *M. anisopliae* (a) NR spectra of clean CaF$_2$ glass slide; (b) NR spectra (control); (c) 3 average SERS spectra. All spectra have been baseline corrected, and shifted vertically for improved visualization.

wavenumber region 600 – 1670 cm$^{-1}$. The distinct band at 659 cm$^{-1}$ ($I_{659}$) scaled at 5.2 x 10$^4$, substantially higher than *B. bassiana*’s enhancement and comparable to *I. fumosorosea*’s enhancement for the selected peak at 1254 cm$^{-1}$

3.2 SERS REPRODUCIBILITY OF ENTOMOPATHOGENIC FUNGI

For future analytical applications of this technology in the quick detection of biocontrol against insect pests, the reproducibility of the entomopathogenic fungal SERS signals recorded is critical. The optimal AgNPs size (60 nm diameter) reflects the reproducibility of *B. bassiana*, *I. fumosorosea*, and *M. anisopliae* SERS spectra. For each EPF investigated, 12 SERS spectra were obtained from different places within the same sample (Fig. 5S). Except for modest fluctuations in intensity at the same wavenumber, there were no discrepancies in the characteristic peaks of the spectra of 18 random places in the same sample, implying that the active substrates fungi-AgNPs were very reproducible. In order to demonstrate quantitatively the consistency of our findings, we chose one notable peak for each studied EPF and calculated the average standard deviation (Av. STD). The Av.STD of the of the selected peak intensities ranges from 5 to 12% (Table 1). For SERS quantitative research, less than 20% variance in SERS intensity between different sites of the active substrate is considered acceptable (Wang et al., 2006).

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Selected bands (cm$^{-1}$)</th>
<th>Av. STD(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBCB 66 <em>Beauveria bassiana</em></td>
<td>1474</td>
<td>11.89</td>
</tr>
<tr>
<td>IBCB 130 <em>Isaria fumosorosea</em></td>
<td>1254</td>
<td>12.21</td>
</tr>
<tr>
<td>IBCB 425 <em>Metarhizium anisopliae</em></td>
<td>659</td>
<td>5.17</td>
</tr>
</tbody>
</table>
3.3 SERS INVESTIGATION OF SPECTRAL CHANGE OF ENTOMOPATHOGENIC FUNGI

The average SERS spectra from the three EPFs investigated are shown in Fig. 5 and the SERS bands have been detected, along with their tentative peak assignments (Table 2). The observed bands were assigned with reference to literature values for some fungi species, common biochemical fungi cell wall components, and reference databases. Over the spectral range of 550 to 1700 cm\(^{-1}\), each filamentous fungus exhibits distinct spectral properties. When compared to other EPF, M. anisopliae has the most unique SERS spectrum and looks like bacterial cells. Weak band intensities, position and peak sharpness distinguish two spectral windows between 550 and 1000 cm\(^{-1}\). The spectral region between 1000 and 1700 cm\(^{-1}\) (fingerprint area) is characterized by strong broad and sharp intensities (Fig. 5a-c). The SERS spectrum of M. anisopliae differs significantly from that of B. bassiana and I. fumosorosea in the spectral window between 550 and 1000 cm\(^{-1}\). We noticed considerable variation in shapes and patterns of the low intensities bands. Two weak bands linked with nucleic acids and membrane lipids (phosphatidylinositol) can be found in the SERS spectra of I. fumosorosea (596 cm\(^{-1}\)) and M. anisopliae (580 cm\(^{-1}\)). The ring breathing nucleic acids such as guanine may be assigned to the sharp intense band at 659 cm\(^{-1}\) in the SERS spectra of M. anisopliae, followed by a very weak band at 692 cm\(^{-1}\), which might be assigned to the ring breathing of tyrosine. Another very weak band at ca. 683 cm\(^{-1}\) (B. bassiana) is probably ascribed to tryptophan. In the SERS spectra of I. fumosorosea, the following low intensity band at 714 cm\(^{-1}\) shifts to 717 cm\(^{-1}\) (M. anisopliae), these bands can be assigned to Flavin adenine dinucleotide (FDA) and riboflavin (RF). Flavins are important coenzymes that play a function in the production of bacterial and fungal cell walls. FDA and RF have been discovered to be effective AgNPs colloids nucleation centers in or out of the cell wall, ensuring that they are close to the AgNPs active site or hot spot (S. Efrima, 2009). The broad weak band at 738 cm\(^{-1}\) (B. bassiana) fluctuates in intensity and shifts to a low intensity band at 749 cm\(^{-1}\) (I. fumosorosea), and a band at 761 cm\(^{-1}\) (M. anisopliae), which are attributed to the vibrational modes of the glycosidic ring from N-acetylglucosamine (NAG) and to the adenine derivatives such as DNA/RNA, thymine (T)/ cytosine (C) (De Gelder et al., 2007). The next weak bands at 790 cm\(^{-1}\) (B. bassiana) which can be also assigned to C or uracil (U), at 811 cm\(^{-1}\) (I. fumosorosea) and at 818 cm\(^{-1}\) (M. anisopliae) which can be linked to unsaturated O-P-O stretching, which can be symmetric or asymmetric such as phosphodiester. The next band emerges at 859 cm\(^{-1}\) (M. anisopliae), however it weakens in intensity shifts to 846 cm\(^{-1}\) (B. bassiana) and to 856 cm\(^{-1}\) (I. fumosorosea), which can be assigned to a fungal cell wall component: C-O-C stretching for chitin (Mohacek-Grosev et al., 2001).

In addition, a strong band at 942 cm\(^{-1}\) (I. fumosorosea) can be seen in the Raman spectra of 1, 3-\(\alpha\)-glucan, in the same spectral region, we observed the prominent broad intense band centered...
at 950 cm\(^{-1}\) in the SERS spectra of *M. anisopliae* which is attributed to carbohydrates such as α or β-glucans, that are typical components comprising the fungal cell wall. In the spectral region between 1000 and 1700 cm\(^{-1}\), we found progressive increases in band intensities from very low to extremely high (fingerprint area). In the case of *M. anisopliae* the band (950 cm\(^{-1}\)) also appears as sharp intense band at 1025 cm\(^{-1}\), and shifts to 1021 cm\(^{-1}\) in the SERS spectra of *B. bassiana*; this variability suggests the possibility of overlapping peak in this region with a complex shape bands attributable to C-C, and C-N stretching modes of carbohydrates such as chitin (Fig. 5a-c).

Figure 5. The average SERS spectra of (a) *B. bassiana*, (b) *I. fumosorosea* (c) *M. anisopliae* using AgNPs and cast as dried thin film onto CaF\(_2\) slide. Experimental conditions: 1% (500 mW), 785 nm excitation, 10 s exposure time. The SERS spectra have been baseline-corrected, normalized and shifted vertically for improved visualization. Each SERS spectrum was averaged from 18 measurements.
### Tentative band assignments from SERS spectra of *B. bassiana*, *I. fumosorosea* and *M. anisopliae*

<table>
<thead>
<tr>
<th>Raman shift-region 550 - 1700 cm⁻¹</th>
<th>B. bassiana</th>
<th>I. fumosorosea</th>
<th>M. anisopliae</th>
<th>Tentative assignment&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>596 (w)</td>
<td>580 (w)</td>
<td></td>
<td>Phosphatidylinositol&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>609 (vw)</td>
<td></td>
<td>C-C twisting mode of Phe (proteins)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>663 (s)</td>
<td>659 (vs)</td>
<td>G, Tyr (ring breathing modes)</td>
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<td></td>
</tr>
<tr>
<td>683 (w)</td>
<td></td>
<td>Tryptophan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>692 (w)</td>
<td></td>
<td>N-acetyl-D-glucosamine, glycoside</td>
<td></td>
<td></td>
</tr>
<tr>
<td>714 (w)</td>
<td>717 (w)</td>
<td>C-S protein, C-H rocking of &gt;CH₂, A, glycoside</td>
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<td></td>
</tr>
<tr>
<td>749 (vw)</td>
<td>761 (w)</td>
<td>Uracil, T, C, ring breathing modes in the DNA/RNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>790 (vw)</td>
<td>811 (w)</td>
<td>ν (O-P-O) RNA, RF, C, Uracil</td>
<td></td>
<td></td>
</tr>
<tr>
<td>818 (w)</td>
<td></td>
<td>Phospodiester, ν(O-P-O) of DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>856 (w)</td>
<td>859 (vw)</td>
<td>ν(C-O-C), e.g. α and β-glucans, chitin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>926 (mw)</td>
<td></td>
<td>Phospodiester, deoxyribose, e.g. β-1,3 glucans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>942 (s)</td>
<td>950 (vs)</td>
<td>δ(C=O), ν (C-O), ring vibration of CH, e.g. chitin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1021 (vs)</td>
<td>1025 (vs)</td>
<td>ν(C-C), ν(C-N) phospholipids, carbohydrates, eg. chitin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1078 (w)</td>
<td>1072 (w)</td>
<td>O-P-O (DNA), ν (C-C) or ν(C-O-C); carbohydrates, e.g. 1,3 glucan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1104 (ms)</td>
<td>1117 (w)</td>
<td>1106 (w)</td>
<td>δ(C-H), Phe (protein assignment)</td>
<td></td>
</tr>
<tr>
<td>1133 (w)</td>
<td></td>
<td>=C-O-C= (unsaturated fatty acids in lipids), gaactomannan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1157 (w)</td>
<td>1157 (vw)</td>
<td>1161 (s)</td>
<td>=C-C=unsaturated fatty acids in lipids, C-N aromatic amino acids</td>
<td></td>
</tr>
<tr>
<td>1192 (vw)</td>
<td>1185 (ms)</td>
<td></td>
<td>δ(C-H), C-O ring, aromatic aminoacids in proteins</td>
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</tr>
<tr>
<td>1223 (vw)</td>
<td>1212 (vw)</td>
<td></td>
<td>Amide III , antisymmetric, ν(PO2)</td>
<td></td>
</tr>
<tr>
<td>1254 (vs)</td>
<td>1237 (w)</td>
<td></td>
<td>Amide III</td>
<td></td>
</tr>
<tr>
<td>1284 (vs)</td>
<td>1295 (w)</td>
<td>1291 (vs)</td>
<td>Amide III (of collagen), T</td>
<td></td>
</tr>
<tr>
<td>1343 (w)</td>
<td>1331 (w)</td>
<td>1331 (w)</td>
<td>A, G, def. (CH); CH₃CH₂ wag, purine bases of nucleic acids</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Tentative assignment.

<sup>b</sup> Phosphatidylinositol.
Table 2 (continued)

<table>
<thead>
<tr>
<th>B. bassiana</th>
<th>I. fumosorosea</th>
<th>M. anisopliae</th>
<th>Tentative assignment&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1376 (w)</td>
<td>1375 (vs)</td>
<td>1374 (s)</td>
<td>T,A,G (ring breathing modes of purines and pyrimides)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1419 (ms)</td>
<td>1426 (w)</td>
<td>1429 (w)</td>
<td>CH₂ sciss (lipids), G,A; COOH</td>
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<tr>
<td>1474 (vs)</td>
<td>1472 (ms)</td>
<td></td>
<td>δ(CH(CH₂)) mode in proteins and lipids</td>
</tr>
<tr>
<td>1518 (w)</td>
<td>1523 (vs)</td>
<td>1490 (s)</td>
<td>def. (CH₂), Lipids</td>
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<tr>
<td>1545 (w)</td>
<td></td>
<td></td>
<td>Aminde II, ν(C=C) in benzenoid ring</td>
</tr>
<tr>
<td>1564 (nw)</td>
<td>1561 (w)</td>
<td></td>
<td>δ(C=C) mode of Phe, e.g. histidine</td>
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<tr>
<td>1595 (w)</td>
<td></td>
<td></td>
<td>A, G, (DNA/RNA); δ(C=C) mode of Phe; C=C (lipid)</td>
</tr>
<tr>
<td>1629 (ms)</td>
<td>1625 (w)</td>
<td>1614 (vs)</td>
<td>Amide I, C=O, plane δ(N-H), ν(C-N), Tyr</td>
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<tr>
<td>1682 (w)</td>
<td></td>
<td>1660 (w)</td>
<td>Amide I of protein</td>
</tr>
</tbody>
</table>

<sup>a</sup> All the assignments are from references: De Gelder J. et al., 2007; Huang E. W., 2010; Malequin K. et al., 2002; Prusinkiewicz M.A. et al, 2012; K.De Gussem et al., 2005; K. De Gussem et al., 2007; Lemma T. et al., 2016.

<sup>b</sup> u, stretching; δ, bending; ρ, rocking; wag, wagging; twist, twisting; sciss, scissoring; def, deformation; as, asymmetric; s, symmetric; C, cytosine; T, thymine, A, adenine; G, guanine; Phe, phenylalanine; Tyr, tyrosine

Table 2). This could be owing to a mismatch between the relative concentrations of aminoacids found in different fungal species’ cell walls and SERS spectra, which have narrow band positions and low intensities. Moreover, the complex symmetrical mode of PO₄²⁻ is composed of DNA and C-C, C-O-C, stretching bands, which were found in the Raman spectrum of carbohydrate molecules including 1, 3-β-glucan and chitin, both of which are frequent components of fungal spores. Chitin makes about 10-20% of the cell wall in Aspergillus species. The chitin material appears to be a fibrillar layer adjacent to the plasma membrane that is thought to perform a structural role in the cell wall’s basal layer (Pietzark et al., 2016). It’s reasonable to assume that the SERS
spectra of fungal cell walls, with putative contributions from metabolic activity and molecular species detached from biochemical structures on the cell surface are influenced by metabolic activity and molecular species. Similarly, at 1104 cm\(^{-1}\) (B. bassiana), a sharp medium band was seen, possible due to bending C-H mode in proteins such as phenylalanine. In the same spectral region, two strong bands were found in two fungal species, the first medium sharp at 1161 cm\(^{-1}\) (M. anisopliae) and the other at 1185 cm\(^{-1}\) (I. fumosorosea). The relative quantity of protein components within their own structural morphological features may influence the adsorption behavior of AgNPs. The M. anisopliae spectrum (Fig.5c) displayed several high intense bands, the first of which strong sharp at 1291 cm\(^{-1}\), was attributed to amide III asymmetric stretching of PO\(_2^+\), most likely from thymine. The amide III vibrational mode's sensitivity and exceedingly intricate features, such as interaction with other vibrational bands, make it difficult to assign the Raman band to specific groups. In I. fumosorosea, this band (1291 cm\(^{-1}\)) becomes more intense and shifts to a very strong band at 1254 cm\(^{-1}\), whereas in B. bassiana, it becomes less intense and shifts to 1284 cm\(^{-1}\). The next prominent signal in the SERS spectra of M. anisopliae appears at 1374 cm\(^{-1}\), which also appears at 1375 cm\(^{-1}\) (I. fumosorosea) in the same spectral region. These bands were assigned to ring breathing modes of purine bases of nucleic acids such as adenine, guanine or thymine. Those signals may be linked to the degradation of nucleic acids and nucleotides caused by cell starvation, which leads in the release of adenine and guanine outside the cells, as documented by Premasiri et al. (2005). In the SERS spectra of M. anisopliae, a weak band at 1419 cm\(^{-1}\) rises to a broad peak at 1490 cm\(^{-1}\). This band increases intensity and shifts to 1474 cm\(^{-1}\) in the SERS spectra of B. bassiana., but it decreases in intensity to 1472 cm\(^{-1}\) in I. fumosorosea. These signals can be ascribed to CH\(_2\) deformation of lipids such as trilinolenin, into the chemical composition of spores, which consist with the spore’s fundamental biological function as an energy reserve for dispersal and reproduction (De Gussem, et al., 2005). The C=C bending mode in benzene ring, amide II, and possibly nucleic acids can be ascribed to the following medium intense band at 1561 cm\(^{-1}\) in the SERS spectrum of M. anisopliae (Fig 5c). In the SERS spectrum of I. fumosorosea this band (1561 cm\(^{-1}\)) appears to have enhanced in intensity and shifted to a sharp band 1523 cm\(^{-1}\), but has declined in intensity to a weak intensity band at 1545 cm\(^{-1}\) (B. bassiana). These signals can be assigned to amide II and C=C bending of phenylalanine. C=O stretching, in-plane modes of N-H bending, and \(\nu\) (C-C) vibrational modes are the most common modes in the amide group. A very strong band at 1614 cm\(^{-1}\) (M. anisopliae) could be ascribed to amide I and possible proteins. Two weak bands at 1629 cm\(^{-1}\) and 1682 cm\(^{-1}\) develop in the SERS spectra of B. bassiana, emerge, and a very low intense band at 1625 cm\(^{-1}\) in the SERS spectra of I. fumosorosea. The dominant secondary structure and the existence of \(\beta\)-plated sheet configuration in these species have been associated to the observed wavenumber.
range 1625-1695 cm\(^{-1}\) (Lemma et al., 2019). Thus, these bands could be ascribed to C=O stretching in conjunction with N-H bending in plane and C-N stretching bands (Fig 5a-c, Table 2).

### 3.4 STATISTICAL DISCRIMINATION

The 2D PCA diagram (PC-1 vs PC-2) displays 75% of the total variance, clearly indicates four distinct clusters that correspond to the three fungi investigated (Fig.6a). PC-1, PC-2, and PC-3 were found to be the most diagnostically significant, accounting for 41.4 %, 33.2%, and 25.4 % of the variation in the data set, respectively. This demonstrates the excellent separation of three distinct clusters, corresponding to: *B. bassiana*, *I. fumosorosea*, and *M. anisopliae*, respectively (Fig 6b).

The bands investigated in detail (section 3.3) are responsible for the variations between the three EPF strains (Fig. 5a-c, Table 2). The loading plot of PC-1, PC-2 and PC-3 in Fig. 6c revealed the variables with the highest loading values are the essential for diagnostic reasons. The loading spectrum, unlike the SERS spectrum, has positive and negative bands, and the frequencies correlate to some of the significant changes in the chemical composition of each EPF studied. The most notable differences among the three EPFs were discovered using SERS spectra and loading graphs from PCs, the bands at ca. 659, 950, 1021,1291, 1375 and, 1474 cm\(^{-1}\) have the most significant variation modes correlate to fungal walls components such as aminoacids, polysacharides (α, βglucans, chitin) and lipids (see Fig.5 and Table 2).

Figure 6. Three PCs (PC-1, PC-2, and PC-3) a) score plot 2D-PCA; b) scores plot 3D-PCA fir the SERS spectra of four fungi investigated in the region (550-1700 cm\(^{-1}\)); c) loding plot for the PCs showing the most significant bands in the spectral window.
These macromolecules that come into direct contact with the AgNPs hot spots, which increases the scattering (Luo et al., 2017). In our experiments we used mixed sample of hyphae and conidia in suspension for sample preparation with AgNPs and SERS measurements to detect three EPF strains. However, we recommend to prepare conidial and hyphae suspensions of EPF samples separately and to standardize the sample reaparation. The use of SERS technique will improve biocontrol agents and almost certainly result in far better products that can compete successfully with their chemical rivals.

4 CONCLUSIONS

We demonstrated the detection and identification of three EPF strains using a SERS technique in conjunction with PCA. The SERS intensity increases up to $7.0 \times 10^4$ when compared to the standard NR. The spectrum's reproducibility was determined by calculating the Av.STD of the selected peak intensities, which ranged from 5 to 12 %, indicating that our findings were statistically reproducibility. The SERS spectra of *M. anisopliae* show increased fluctuation and shift wavenumber, particularly in the 550-1000 cm$^{-1}$ spectral range, and differ significantly from those of *B. bassiana*, and *I. fumosorosea*, indicating molecular composition heterogeneity among the three fungal strains. The presence of phospholipids, carbohydrates such as chitin and $\alpha$, $\beta$-glucans, and all three amide groups I, II and III detected in the SERS spectra of the three EPF studied is noteworthy, these biomolecules are common components of the cell wall and spores of fungal species or molecular species detached from the cell surface of EPF. In the SERS spectral area (550-1700 cm$^{-1}$), the PCA scores obtained by PC-1, PC-2, and PC-3 values accounted for almost 99 percent of total variance, validating the classification of three separate clusters, each corresponding to entomopathogenic fungi studied.

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REFERENCES


