MALDI TOF MS-profiling: Applications for bacterial and plant sample differentiation and biological variability assessment

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A B S T R A C T

In this study, we evaluated the potential use of MALDI-TOF MS Profiling for the differentiation of biological samples submitted to different treatments. We compared the bacterium Xanthomonas campestris pv. campestris (Xcc), grown in culture medium and in vivo (recovered from the plant). Plant samples were also analyzed and included explants at different somatic embryogenesis (SE) stages, as well as leaves from Brassica oleracea and Arabidopsis thaliana inoculated with Xcc, at different time points. The results showed that bacteria and highly divergent plant samples, such as those from embryogenic stages, can be unequivocally differentiated and the clustering was in accordance with proteomic analysis performed by 2-DE. These results show an important application of MALDI-TOF MS Profiling to select and prioritize samples to be analyzed prior to more complex approaches including transcriptomics and proteomics. We also show that in plant-pathogen interactions, when more subtle differences are obtained, the main contribution of MALDI-TOF MS Profiling is in the assessment of experimental variability. This is relevant since reproducibility is a challenging issue when dealing with complex experimental conditions such as plant-pathogen interactions. We propose the use of MALDI-TOF MS Profiling to aid researchers in minimizing experimental variability unrelated to the condition being analyzed.

Significance: MALDI-Profiling offers an inexpensive, rapid and reliable approach for investigating the protein profile to assess sample differentiation and experimental variability in microorganisms and plants and can be highly useful to analyze samples prior to more complex and expensive techniques such as proteomics and transcriptomics.

1. Introduction

Important advances in mass spectrometry and data analysis softwares have been achieved in the past few years, allowing important contributions for scientific development in different areas. MALDI-TOF MS (Matrix-assisted laser desorption and ionization time-of-flight mass spectrometry) is a powerful technique to achieve protein and peptide fingerprints. In the last decade, studies have shown the use of mass spectrometry for the differentiation of bacteria by combining MALDI-TOF MS with a software containing a protein profile database and comparison algorithms, which allows the identification of microorganisms in the species level [1–3]. Since then, MALDI-TOF MS Profiling (MALDI-Profiling) has been used to identify several microorganisms, including Gram-positive and Gram-negative bacteria [4–6]. The main application of this technique is for bacterial identification in clinical samples and has been routinely used for this purpose [7–9]. There are few studies reporting the use of this method aiming at the identification of microorganisms of environmental importance, such as plant-interacting bacteria or fungi [10,11].

Previously, we published a short review and perspective paper discussing the visionary applications of MALDI-Profiling in sample differentiation [12]. In the current work, we show a large amount of experimental data achieved from analyses of several biological samples, mainly plant-derived materials, and demonstrate that MALDI MS protein profiling is a powerful method for sample differentiation, as well as for biological variability assessment among experimental treatments.

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2. Materials and methods

2.1. Biological samples analyzed by MALDI-profiling

2.1.1. Xanthomonas campestris pv. campestris grown in vitro and in vivo

We used the bacterium Xanthomonas campestris pv. campestris (Xcc), isolate Xcc 51, obtained from the culture collection of Embrapa Hortaliças-CNPH, Brazil. Xcc was cultured in vitro in the rich medium Nutrient Yeast Glycerol (NYG) according to Daniels et al. [13]. In vitro samples were obtained by recovering Xcc from the host plant Brassica oleracea susceptible (Kenzan) to Xcc, according to Mehta & Rosato [14], with some modifications. B. oleracea leaves were inoculated with Xcc (OD = 0.6) and collected at 24 and 48 h after inoculation (hai). Collected leaves were cut with a razor blade and maintained in a Petri dish (OD = 0.6) and collected at 24 and 48 h after inoculation (hai). Collected leaves were washed in distilled water and fragments measuring about 2.5 cm × 2.5 cm were collected from three individual plants from each cultivar at 24 and 48 h after inoculation (hai), representing the beginning of infection. Three biological replicates were performed for each sample.

2.1.2. Somatic embryogenesis samples of oil palm

Immature leaves of an oil palm interspecific F1 hybrid of Elaeis oleifera × E. guineensis (B352933), non-responsive to somatic embryogenesis (SE), were collected and submitted to the disinfectations with 70% ethanol and 2.5% sodium hypochlorite for 30 min. After that, leaves were washed in distilled water and fragments measuring about 1 cm² were submitted to the callus induction process in MS culture medium [15], supplemented with 30 g L⁻¹ sucrose, 0.5 g L⁻¹ glutamine, 0.5 g L⁻¹ hydrolysed casein, 2.5 g L⁻¹ activated charcoal, 450 µM 4-amino-3,5,6-thri chloride picolinic acid (Picolram) and solidified with 2.5 g L⁻¹ of Phytagel (Sigma), according to Balzon et al. [16]. During this process (maintained for 180 days), leaf explants were stored in the dark at 25 ± 2 °C. Over this period, the material was randomly collected at 0, 14, 90 and 150 days of induction (doi) in biological triplicates (R1, R2 and R3), macerated in liquid nitrogen, stored at −80 °C and used for protein extraction.

2.1.3. Brassica oleracea inoculated with X. campestris pv. campestris

Cultivars of B. oleracea resistant (União) and susceptible (Kenzan) to Xcc were used in this study. At 45 days after sowing, plants were inoculated with Xcc suspension (A₅₀₀nm = 0.6) or 0.85% NaCl (control condition). Xcc was cultured in the rich medium NYG and used for inoculation of the plants. The abaxial surface of the leaves was infiltrated with bacterial suspension using a syringe. Leaves were collected from three individual plants from each cultivar at 24 and 48 h after inoculation (hai), representing the beginning of infection. Three biological replicates were performed for each sample.

2.1.4. Arabidopsis thaliana inoculated with X. campestris pv. campestris

A. thaliana seeds were sterilized in 70% ethanol and 0.05% Triton X-100 and sown in MS medium [15] supplemented with 10 g L⁻¹ sucrose and 8 g L⁻¹ agar. Plates were maintained in a controlled growth chamber with 12:12 h photoperiod and 22 ± 2 °C. At 30 days after sowing, plants were sprinkled with Xcc 51 solution (OD = 0.1) in sterile water and only with water (control condition). At 5 days after inoculation (dai), stressed plants (confirmed by typical black rot symptoms) were collected, macerated in liquid nitrogen and used for protein extraction.

2.2. Protein extraction

Approximately 0.2 g of plant tissue and bacterial cells in vivo (recovered from plant leaves) and in vitro (1.5 mL of culture at OD = 0.8) were used for protein extraction. Total proteins were extracted according to Mot and Vanderleyden [17], using 750 µL of extraction buffer (0.7 M sucrose, 0.5 M Tris-HCl, 30 mM EDTA, 0.1 M KCl and 40 mM DTT) and the same volume of buffer-saturated phenol. Proteins were precipitated in 0.1 M ammonium acetate in methanol and washed with 80% acetone.

2.3. MALDI-profiling analysis

Extracted proteins were solubilized in 70% formic acid and acetonitrile following standard procedures. The instrument was calibrated with the Bruker Bacterial Test Standard (Bruker Daltonics) prior to the analyses. A total of 1 µL of each protein sample was deposited onto a MALDI target plate (Bruker MSP 96 target polished steel BC). After air-drying, 1 µL of a saturated solution of α-cyano-4-hydroxycinnamic acid matrix (according to the original Biotyper method) was deposited over each sample. The spectra were acquired using a MicroFlex instrument (Bruker Daltonics) and the data were analyzed using the Biotyper software version 3.0 (Bruker Daltonics), using standard procedures. Main spectra profiles (MSP) were generated from the samples and the final data were represented and visualized as dendrograms based in Euclidean distances. MSP dendograms obtained by MALDI Biotyper approach are based in average mass spectral profiles from several individual mass spectra which are thus compared by hierarchical clustering routines. It allows showing molecular groupings that could be associated to similar chemical characteristics. A total of 12 technical replicates and 3 biological replicates were analyzed, totaling 36 acquisitions for each condition. The mass range used for acquisition was between 2000 and 20,000 m/z and standard peak-picking algorithm (the same commonly used for microorganisms). The standard mass range used by the Biotyper software for analyses was between 3000 and 15,000 m/z. We performed the alignment of the MS spectra using FlexAnalysis software in order to identify the differential protein peaks.
Fig. 2. Overview of the results obtained by MALDI-TOF MS Profiling and 2-DE from an interspecific hybrid between *Elaeis oleifera* and *E. guineensis*. (A) Dendrogram generated from oil palm at different days of induction (DOI) during somatic embryogenesis at 0, 14, 90 and 150 DOI. (B) 2-DE Maps showing the protein profile during somatic embryogenesis at 0, 14, 90 and 150 DOI and the corresponding representative mass spectra. A total of three biological replicates were analyzed (R1, R2 and R3).
Fig. 3. Dendrograms generated from *B. oleracea* plants. Susceptible (SBI) and resistant (RBI) plants analyzed 24 h after inoculation (hai) with Xcc and compared to control susceptible (SBC) and resistant (RBC) plants. A total of three biological replicates were analyzed (R1, R2 and R3).

3. Results and discussion

The results obtained in this study revealed an important and novel application of MALDI-Proﬁling; we show that this technique can be used for protein proﬁle differentiation of diverse samples, including bacteria as well as plants.

Initially, we compared the protein proﬁle of the bacterium *X. campestris pv. campestris* (Xcc) under two different growth conditions: *in vivo* (Xcc recovered from inoculated *B. oleracea* leaves) and *in vitro* (Xcc cultured in rich medium NYG). We chose to begin our assays with bacteria, since they are simpler organisms and we had prior information from our previous studies that both growth conditions resulted in a highly distinct protein proﬁle [18].

Interestingly, the dendrograms obtained revealed a clear difference between the protein proﬁles of both conditions. This difference was observed for Xcc recovered 24 and 48 hai *in vivo* and *in vitro* (Fig. 1A and B and Fig. S1). It is important to note that MALDI-Proﬁling depicts only a fraction of the entire protein proﬁle, since a limited m/z range is detected (2000-20,000). Therefore, care must be taken when extending/extrapolating the results to the entire proteome proﬁle. However, the general profile observed in the 2-DE analysis was very different, even though differential proteins in the m/z below 20,000 were not observed [18]. Moreover, when the same conditions *in vivo* susceptible (named REK in Santos et al. [19]) and NYG) were analyzed by a more sensitive proteomic approach (LC-MS/MS), 105 differential proteins were identiﬁed, out of which 31 were in the m/z range below 20,000 [18]. Although plant proteins (a total of 195) were also detected, which is the major drawback of the *in vivo* analysis, only 10 proteins in the m/z range below 20,000 were observed in REK. These results show that MALDI-Proﬁling could be used as an efﬁcient method to differentiate the protein proﬁle of plant pathogenic bacteria submitted to different growth conditions.

To verify if this approach could also be applied to more complex samples, such as plant material, we analyzed the protein proﬁles of different somatic embryogenesis stages in an interspeciﬁc F1 hybrid between *E. oleifera* and *E. guineensis* (B3S2933). The initial stages at 0 and 14 days of induction (doi) and later stages at 90 and 150 doi were compared. The generated dendrogram revealed three clusters, showing that the most distinct protein proﬁle was that at 0 doi, followed by 14 doi. The cluster also revealed that the proﬁles at 90 and 150 doi were quite similar and therefore these samples were grouped together (Fig. 2A). Furthermore, the generated peaks were in accordance to the dendrogram generated showing that the proﬁles at 0 doi, followed by 14 doi were the most distinct and that the proﬁles at 90 and 150 doi were quite similar (Fig. 2 and Fig. S2).

These results could be conﬁrmed by 2-DE analysis, which showed a clear distinct proﬁle at 0 and 14 doi and similarity between the proﬁles from 90 and 150 doi in the same m/z range detected by MALDI-Proﬁling (Fig. 2B, Fig. S2 and Fig. S3). Therefore, we conclude that MALDI-Proﬁling can also be used to differentiate complex samples such as somatic embryogenic tissues from plants. As mentioned above, MALDI-Proﬁling reveals only a fraction of the protein proﬁle, however, in general, the results obtained are in accordance with the 2-DE proﬁle, showing that 0 doi has the most distinct proﬁle, followed by 14 doi.

An important outcome of this analysis is the knowledge of the protein proﬁle prior to more complex studies such as transcriptomic and proteomic analyses. The selection of the sampling point to be analyzed is often a challenge, since we do not know the exact time the cell is responding to environmental changes. Moreover, when more complex techniques are used, the number of samples is usually kept to a minimum, due to experimental costs and intense bioinformatic analysis required. MALDI-Proﬁling can help researchers assess sample variability and use the information obtained to select and prioritize the samples to be further analyzed by more complex and costly techniques.

We were also interested in differentiating samples during plant-pathogen interaction. Therefore, we compared *B. oleracea* plants in the control condition and inoculated with Xcc in 3 biological replicates grown under greenhouse conditions. Unexpectedly, the MALDI-
Profiling dendrogram showed a tendency in clustering according to the biological replicate, not globally differentiating the stress condition (Fig. 3). When working with plants in greenhouse, although considered as a “controlled condition”, there are also a number of variables, such as temperature, humidity, light incidence, pests, etc. which cannot be truly controlled. This variability in the replicates are often obtained in several plant-pathogen interactions (e.g. virus, bacteria and nematode inoculations), although not often reported in the literature. Variability is usually overcome by a higher number of replicates or by more stringent parameters in statistical analyses. Another possibility is in vitro growth, in which environmental conditions can be better controlled. To test our hypothesis, we decided to test in vitro conditions using Arabidopsis thaliana. Plants were grown in Petri dishes, inoculated with Xcc and compared to non-inoculated plants. We used a total of 11 replicates split into two independent experiments (5 replicates in one experiment and 6 in another). The dendrograms obtained showed a clear differentiation between stressed and unstressed plants. Surprisingly, it also revealed a separation of the groups according to the experimental replicate (Fig. 4).

These results raise the question of how many biological replicates are needed in order to get sound results under greenhouse or in vitro conditions. It is common sense that when complex techniques such as shotgun proteomic and transcriptomic approaches are employed, usually the number of replicates is kept to a minimum (around 2–3) due especially to the costs. Another frequent difficulty is the variability observed among the biological replicates in plants. It is systematically a challenge to get reproducible (statistically significant) expression profiles in all replicates (usually 3), which is due to biological variability only discovered when the transcriptomic or proteomic analyses are concluded. We can infer that the variability may be due to environmental conditions but it can also be due to failure in the applied treatment. Since samples are normally obtained at early stages during infection or drought treatment, there is no way of knowing if the stress was successfully applied to the plant, since the effects are only seen at later stages. Therefore, normally, additional plants are kept for longer periods to confirm the stress applied, although the samples are usually collected from different plants. For example, in experiments related to phytonematode infection, 1–2 months are necessary to confirm the success of an infection. Another possibility could be the timing of the response to the pathogen. It is not possible to have complete control over the exact amount of inoculums in each plant and to synchronize their response to the pathogen. These variables are minimized (not eliminated) by usually pooling 3 or more plants together to form one biological replicate.

4. Conclusions

Based on the results obtained in this study, we propose the use of MALDI-Profiling to aid researchers decide and select their samples based on the homogeneity and successful differentiation prior to more complex and costly analyses. Also, the variability among the replicates can be obtained and contribute to better evaluate the experiment. Thus,
we believe that MALDI-Profiling offers an inexpensive, rapid and reliable approach for investigating the expression profile, sample differentiation and experimental variability and can be highly useful to support further analysis of the samples using more complex and expensive techniques. Moreover, specifically in the case of plant-pathogen interaction, where subtle differences are obtained in gene and protein expression, an assessment of experimental variability can also be obtained.

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Author contributions statement

DG Ribeiro; LST Carmo; IR Santos performed protein extraction and sample preparation; LP Silva performed mass spectrometry analysis; OB Oliveira Neto grew the plants and performed inoculations; RF Almeida and JES Pereira prepared the somatic embryogenic material; A Mehta lead the work and prepared the manuscript. All authors reviewed the manuscript.

Declaration of Competing Interest

Authors have no competing interests to declare.

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