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Short Communication

MALDI-MSI method for the detection of large biomolecules in plant leaf tissue

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Abstract

In this study we describe a method for the detection of biomolecules (in the polypeptide *m/z* range) directly from the surface of plant leaves by using Mass Spectrometry Imaging. The plant-pathogen interaction between *Arabidopsis thaliana* and the bacterium *Xanthomonas campestris* pv. *campestris* was analyzed by comparing infected and non-infected leaf discs submitted to mass spectrometry. The total surface area of ion distribution was calculated for both samples, revealing 23 ions, out of which 3 showed statistical significance. Although these ions were not identified, the results showed that this approach can be successfully applied for the detection of potential polypeptide biomarkers directly on leaf tissue, which is a major challenge in MALDI-Imaging studies.

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Keywords: Mass spectrometry imaging; Plant-pathogen interaction; Protein biomarkers; *Xanthomonas campestris* pv. *campestris*; *Arabidopsis thaliana*

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Introduction

Advances in mass spectrometry analyses and the development of new software have made important contributions to the scientific development. In the last two decades, this technology has been applied to map and measure ions from biomolecules, such as proteins, lipids and other biological compounds, directly on the surface of tissue samples [1-4]. Matrix assisted laser desorption ionization-time of flight (MALDI-TOF) instruments have been commonly employed in mass spectrometry imaging (MSI) analyses and several studies have shown the use of this technique for the investigation of biomarkers in clinical studies [5-8]. Although this approach has also been applied to plant tissues revealing the spatial distribution of metabolites and proteins [9,10], its application is limited in studies involving plant-pathogen interaction. Some of these studies have focused on the detection or the distribution of defense molecules [11-15]; however, most protocols described so far, especially for the detection of proteins, involve cryosectioning [16-18].

Improvements in instrumentation and sample preparation have helped establish MALDI-MSI technique as a good choice for visualization of biomolecules in biological tissues [19]. Moreover, it has been widely used as a complementary tool in other classic studies, such as histology and molecular analyses [20]. Another application for MALDI-MSI is the differential analysis of healthy and infected tissues. Studies have applied this technique in mice models to identify inflammatory protein masses in tissues infected with *Staphylococcus aureus* [21] or during visceral leishmaniasis [8], however in plants it has been rarely used. Here, we describe a method for the detection of biomolecules (in the polypeptide *m/z* range) directly from the surface of leaves. We used as a model for this work the interaction between *Arabidopsis thaliana* and the bacterium *Xanthomonas campestris* pv. *campestris* (Xcc), the causal agent of black rot disease in cruciferous plants, in order to verify if differential proteins could be identified. We show the potential application of this tool in the detection of biomarkers associated with the infection caused by the bacterium, which could help in the development of strategies for pathogen detection and control.

Materials and methods

Sample preparation

Plants of *Arabidopsis thaliana* (Columbia-0 ecotype) and the *Xanthomonas campestris* pv. *campestris* isolate 51 (Xcc 51), obtained from the culture collection of phytopathogenic bacteria of Embrapa Hortaliças, Brasilia, DF, Brazil, were used. *A. thaliana* seeds were sterilized in a solution of 70% ethanol/0.05% Triton X-100, dried using a filter paper and transferred to plates containing nutrient agar medium [Murashige and Skoog (MS) basal salt mixture (SIGMA-ALDRICH), 1% sucrose and 0.8% agar]. The plates were incubated in a growth chamber with 12 h light: 12 h dark cycles at a temperature of 22-24°C.

Seedlings of *A. thaliana* (3-4 week old) were inoculated with Xcc suspension (A_{600} = 0.1) prepared in distilled water. For the control condition, leaves were inoculated only with distilled water. Five days after inoculation, infected and mock-inoculated leaves were used for MALDI-MSI analysis. Leaf discs from 5 biological replicates were cut with the aid of a perforator (8 mm in diameter) and immersed in isopropanol/methanol 50% (v/v) solution for 5 s three times.

MALDI-MSI analysis

After drying in a vacuum desiccator for 5 min, leaf discs were fixed onto a MALDI target plate using double-sided adhesive/conductive tape. Then, 15 mL of a matrix solution (5 $mg.mL^{-1}$ sinapinic acid in 50% ACN and 0.3% TFA) was deposited on the plant tissue using an artistic airbrush spraying device (Lee Pro Tools 601115) connected to nitrogen gas. For that, samples were placed 30 cm away from the spray jet and cycles during 3 s of spraying were intercalated with a 30 s drying time. After airbrush spraying, samples were dried under vacuum for 30 min followed by a final application of matrix solution (10 µL/leaf disc) performed with the aid of a pipette to ensure complete leaf disc coverage, as described previously [22]. After complete drying, the leaf discs were submitted to MALDI-TOF-MSI acquisition of proteins using an UltraFlex III mass spectrometer (Bruker Daltonics).

All mass spectra were acquired in the positive linear mode with laser (solid state) intensity fixed at 30% and using steps of 350 µm distance among the acquisition points and ablation diameter of 100 µm. Automatic scanning procedure was performed using FlexImaging 4.0 software (Bruker Daltonics). Global spectra were generated for each image from 200 laser shots at each position in the global maps. Ions were detected in the mass range of polypeptides, which is between *m/z* 1,740-25,000. External calibration was performed using a calibrant mixture (protein calibration standard I - Bruker) applied on a leaf disc, which was not used for subsequent analysis.

Image analysis

ImageJ software (1.50i 64-bit) was used to measure the area of ionic distribution obtained from FlexImaging™ software (Bruker). Colored images of each protein distribution were normalized using ImageJ software. The different pixels of black color were saturated, displaying white color and the areas were calculated. The data were used to evaluate the percentage occupied by the protein ion in comparison with the total leaf disc area. The mean and standard deviation were calculated for each ion and Student's t-test with equal variances was used to identify significant changes on ionic distribution ($p \leq 0.05$).

Results

In this study we were interested in analyzing ion distribution directly on the leaf surface during a plant-pathogen interaction in an attempt to identify potential biomarkers associated to infected tissues. We used the model plant *A. thaliana* infected with *X. campestris* pv. *campestris* in order to optimize the detection of ions on the protein range using MALDI-MSI. Five days after inoculation (DAI), infected plants began to show chlorosis and V-shaped lesions, typical of black rot disease, while at 7 DAI severe symptoms could be observed (Figure 1). Since inoculated leaves at 7 DAI were very fragile, only leaves at 5 DAI were used and compared to the control condition. A total of 23 ions presented a minimum intensity threshold of 3, showing that this approach can be successfully applied for the detection of polypeptides directly on leaf tissue (Figure 2).

When the Student's t-test was applied, out of the 23 ions, 3 showed statistical significance (Figure 2). It is well known that MALDI-MSI presents variability in detection and therefore a high number of replicates is desirable. However, the amount of time necessary for data acquisition and processing also increase substantially. By analyzing the individual ion data (Table S1), we could observe that although only ions at *m/z* 2620, *m/z* 3246 and *m/z* 4692 were statistically validated, other ions could also be further investigated as interesting candidates to confirm their differential expression trend by other methods.

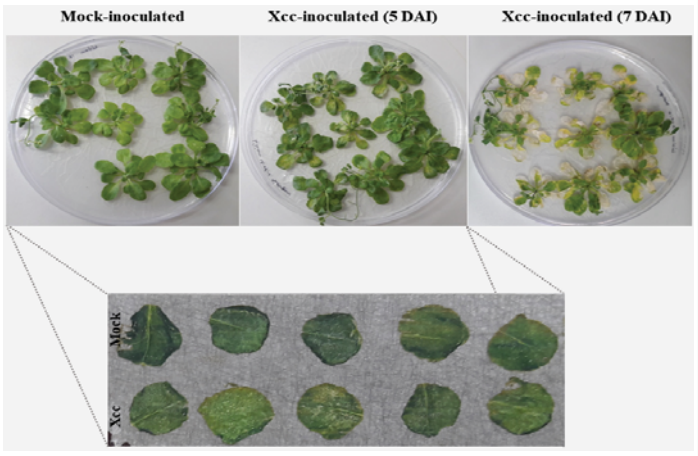

Figure 1: Symptoms in *A. thaliana* plants (3-4 week old) inoculated with *Xanthomonas campestris* pv. *campestris* (Xcc) showing chlorosis and necrosis at 5 and 7 days after inoculation (DAI), respectively.

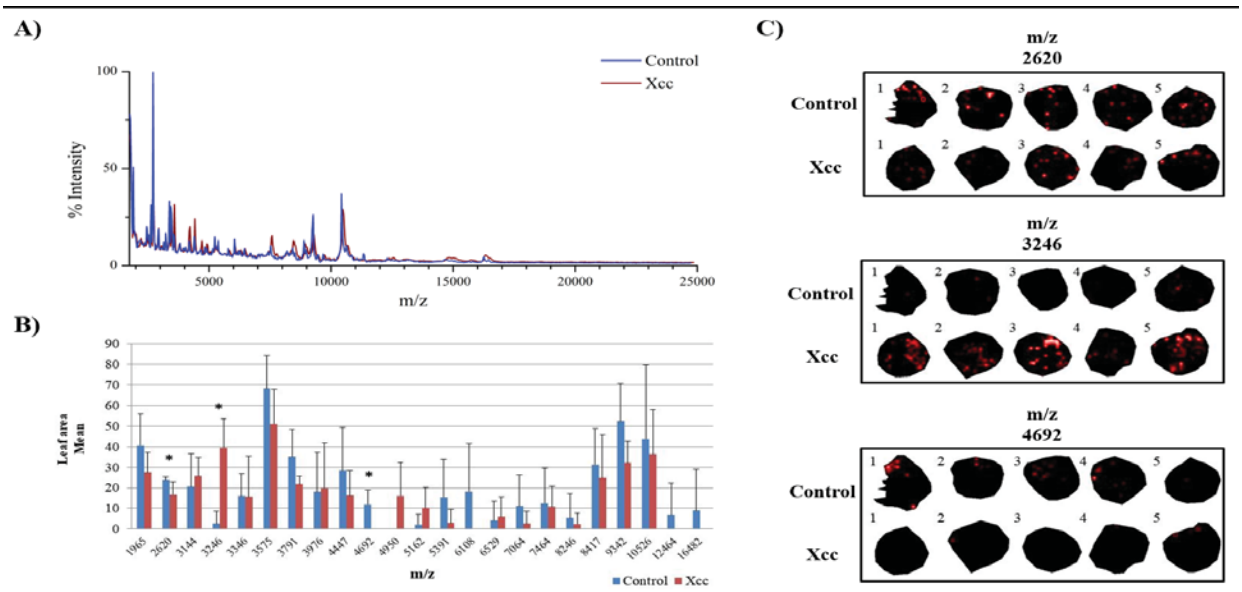

Figure 2: Potential protein biomarkers revealed in leaves of *A. thaliana* infected with *Xanthomonas campestris* pv. *campestris* (Xcc) using MALDI-MSI approach. A) Overlapping image of the global mass spectrum obtained from the acquisitions of MALDI-MSI in the control and Xcc inoculated leaves. B) Graph of the leaf area mean for each ion. (*) Statistically validated ions by Student's t-test (p-value ≤ 0.05). C) Ions distributed in leaf discs inoculated with Xcc and in the control condition. The numbers 1, 2, 3, 4 and 5 represent the biological replicates.

Discussion

The use of MSI in plants has been reviewed by several authors [23-25], however few reports focused on plant-pathogen interactions. Previous studies have successfully revealed the tissue distribution of biological compounds, such as hesperidin that can be associated to defense mechanisms [15], as well as peptides/proteins in infected plants [26]. However, the detection of large biomolecules (*m/z* 1740-25000) directly from plant tissue is a challenge. In this study we present a simple method, optimized for the detection of large biomolecules directly from leaf tissue, without the need for cryosectioning. Our results show that MALDI-MSI approach can be a powerful tool for leaf-pathogen interaction studies, allowing the rapid detection of differentially abundant ions, which contributes for future studies aiming at the identification of proteins associated with defense and resistance in plants. The main contribution of this approach is that the spatial distribution of the detected ions is preserved, unlike other strategies applying multiple preparation steps, which may result in spatially displaced ions. For the first time, potential biomarkers associated with the infection caused by a pathogen are detected directly in leaf tissue by means of this technology. After further validation of these differential protein candidates by other methods, such as RT-qPCR, the corresponding genes can be used as targets for knockout or overexpression in plants aiming at the development of resistant cultivars or for the detection of infected plants.

Author contributions: LTS Carmo and DG Ribeiro performed the plant inoculation experiments and preparation of leaf tissue and wrote the manuscript. EA Barbosa and LP Silva performed the mass spectrometry analyses. A Mehta designed and supervised the experiments.

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Supplementary Table S1: Area of ions obtained in five leaf discs of *A. thaliana* using ImageJ software.

Ions	RC1	RC2	RC3	RC4	RC5	Mean	Standard Deviation	RX1	RX2	RX3	RX4	RX5	Mean	Standard Deviation	Student's t-test (*p* - value)
1965	27.03	46.75	22.48	48.72	58.68	40.73	15.36	28.64	39.27	18.97	33.90	16.93	27.54	9.56	0.14
2620*	22.73	22.99	24.42	22.82	26.25	23.84	1.51	17.06	9.23	26.16	15.79	15.29	16.71	6.09	0.03
3144	0.00	22.49	12.25	24.91	43.29	20.59	16.06	30.94	38.86	22.40	16.10	19.86	25.63	9.19	0.56
3246*	0.00	0.01	0.00	0.01	13.51	2.71	6.04	47.73	34.37	46.82	17.42	51.53	39.57	13.97	0.00
3346	0.00	12.44	16.31	24.52	26.85	16.02	10.71	16.48	47.94	0.01	0.00	13.69	15.62	19.60	0.97
3575	55.85	54.24	59.93	86.19	85.05	68.25	15.99	58.84	76.35	35.97	44.86	39.19	51.04	16.64	0.13
3791	15.66	28.00	37.99	47.51	46.43	35.12	13.41	28.13	21.55	20.55	17.87	20.36	21.69	3.85	0.06
3976	0.00	0.01	14.66	38.57	37.33	18.11	19.08	0.01	47.55	13.15	0.00	37.95	19.73	21.95	0.90
4447	0.01	23.96	44.82	52.32	21.33	28.49	20.72	25.82	7.98	28.31	0.01	19.47	16.32	12.04	0.29
4692*	16.02	10.71	16.84	15.80	0.00	11.87	7.06	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.01
4950	0.01	0.01	0.01	0.01	0.00	0.01	0.00	0.01	37.62	0.01	20.09	23.20	16.19	16.18	0.06
5162	0.00	0.00	0.01	10.80	0.01	2.17	4.83	15.37	0.00	22.71	12.86	0.01	10.19	9.98	0.14
5391	9.24	0.00	42.16	26.01	0.00	15.48	18.31	14.65	0.00	0.00	0.00	0.01	2.93	6.55	0.19
6108	17.07	0.00	17.71	56.50	0.00	18.26	23.08	0.01	0.00	0.00	0.00	0.01	0.00	0.00	0.11
6529	0.00	0.00	0.00	20.90	0.00	4.18	9.35	22.00	8.23	0.00	0.00	0.00	6.04	9.60	0.76
7064	0.01	10.21	0.01	36.89	8.54	11.13	15.15	13.21	0.01	0.00	0.00	0.00	2.64	5.91	0.28
7464	0.00	0.01	31.03	31.20	0.00	12.45	17.04	15.59	0.00	21.21	0.01	17.00	10.76	10.03	0.85
8246	0.00	0.00	0.01	26.54	0.00	5.31	11.87	0.00	0.00	0.01	0.00	12.07	2.42	5.40	0.63
8417	11.24	15.36	45.68	50.18	34.04	31.30	17.52	24.16	35.24	11.24	0.01	53.41	24.81	20.78	0.61
9342	52.84	32.71	67.49	73.10	36.20	52.47	18.07	49.82	28.11	25.98	23.79	32.57	32.05	10.45	0.06
10526	0.00	12.85	66.75	83.48	55.62	43.74	35.77	60.06	54.82	18.52	11.72	36.59	36.34	21.37	0.70
12464	0.00	0.00	0.01	34.55	0.00	6.91	15.45	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.35
16482	0.00	0.00	0.01	44.82	0.00	8.97	20.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.35

The final value of the area was calculated using the ratio of the absolute value occupied by each ion in relation to leaf disc area. A total of 5 biological replicates were used.
RC: number of replicates (control condition).
RX: number of replicates (inoculated with *Xanthomonas campestris* pv. *campestris)*
*Three protein ions showed a statistically significant differential profile ($p \leq 0.05$).
Protein ions highlighted in the table represent the ions statistically validated ($p \leq 0.05$) when the two most discrepant values were removed.