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MALDI-TOF MS to identify the pineapple pathogen *Fusarium guttiforme* and its antagonist *Trichoderma asperellum* on decayed pineapple

Cledir Santos[1] · **José A. Ventura**[2,3] · **Hélcio Costa**[2,3] · **Patrícia M. B. Fernandes**[3] · **Nelson Lima**[4,5]

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Abstract Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) has been used to identify some *Fusarium* and *Trichoderma* species, but early detection of fungal diseases by this technique has not yet been fully addressed. In this study, MALDI-TOF MS was tested to identify *F. guttiforme* on pineapple side shoots in situ. The efficacy of filamentous fungi for controlling fungal diseases is well documented. However, there is uncertainty whether the biocontrol agent is out growing the pathogen sufficiently to be identified. In this paper, a multistep identification of a plant pathogen (*F. guttiforme)* and its antagonist (*T. asperellum)* using MALDI-TOF MS is demonstrated.

Keywords Fungal ribosomal proteins · Fungal early detection · MALDI-TOF MS · Pineapple

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✉ Cledir Santos
cledir.santos@ufrontera.cl

[1] Department of Chemical Sciences and Natural Resources, Universidad de La Frontera, Av. Francisco Salazar 01145, Temuco 4811-230, Chile

[2] Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural (INCAPER), Rua Afonso Sarlo, 160 - Bento Ferreira, Vitória, ES, Brazil

[3] Núcleo de Biotecnologia, Universidade Federal do Espírito Santo, Vitória, ES, Brazil

[4] CEB-Centre of Biological Engineering, Micoteca da Universidade do Minho, University of Minho, Campus of Gualtar, Braga 4710-057, Portugal

[5] Programa de Pós-Graduação em Microbiologia Agrícola, Universidade Federal de Lavras, Campus Universitário, Lavras, MG, Brazil

Introduction

Many *Fusarium* species are pathogenic to a variety of crops (Leslie and Summerell 2006). Diagnostic methods should be rapid and efficient to detect plant pathogens preferably without the need to isolate and grow it on artificial media. Rapid methods available for identifying several *Fusarium* species or complexes include PCR-based assays as discussed elsewhere (Jurado et al. 2005, 2006; Kuzdraliński et al. 2014; Mishra et al. 2003). Pineapple, *Ananas comosus* var. *comosus*, is an important and profitable fruit that is subjected to the occurrence of various diseases, mostly in regions of tropical climate. Fusariosis is a major disease of pineapple causing 30 to 40 % fruit losses fruit and up to 20 % loss in propagative material (Ventura and Zambolim 2002). Infection occurs through the side shoots of pineapples. An early detection of the disease, e.g., prior to harvest, could allow farmers to reduce inoculum by harvesting infected fruit, thus avoiding cross-contamination (Bauriegel et al. 2011). PCR-based methods are available to identify *Fusarium* species (Jurado *et al.* 2005, 2006; Kuzdraliński *et al.* 2014) but more sensitive and cost-effective methods are needed in detection schemes targeting early detection of the pathogen. Successful suppression of *Fusarium* infection in many crops with application of different species of *Trichoderma* as antagonists has been demonstrated (Sundaramoorthy and Balabaskar 2013). However, rapid monitoring of the effectiveness of such control systems has not been developed.

Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) emerged in the late 1980s to investigate molecular masses of organic compounds through a soft ionisation of molecules resulting in minimum fragmentation (Tanaka et al. 1988). MALDI-TOF MS has proven useful for the identification of filamentous fungi at either the species or strain level (Dias et al. 2011; Rodrigues et al. 2011; Santos et al. 2010).

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Intact fungal cells can be analysed as a fingerprint of the profile of organic compounds. The remarkable reproducibility of this technique is based on the measurement of highly abundant organic chemical compounds, which are observed on the mass spectra as very specific ion peaks and used as biomarkers for fungal identifications. Ribosomal proteins are the main chemical compounds used for this purpose. The ions generated by the analysis of these large and natural chemical molecules are observed in a mass range between 2 and 20 kDa. Also, compounds such as polysaccharides, lipids, phospholipids, chitin, among others, can similarly be found in the fungal cell. All of these are very useful in fungal identifications by mass spectrometry (Kemptner et al. 2009; Marvin et al. 2003; Rodrigues et al. 2011; Santos et al. 2010).

Studies have demonstrated the usefulness of MALDI-TOF MS for the identification of species of *Fusarium* and *Trichoderma* (De Respinis et al. 2010; Dong et al. 2009a, b; Kemptner et al. 2009). However, its use for the early detection of filamentous fungi infecting agriculture commodities was not fully addressed and data is lacking in the literature. Furthermore, successful suppression of *Fusarium* in many crops via application of different species of *Trichoderma* as antagonists has been demonstrated (Sundaramoorthy and Balabaskar 2013). However, no method has been developed to quickly monitor the antagonist-pathogen in situ interaction. Hence, this study aimed to evaluate the use of MALDI-TOF MS to identify *F. guttiforme* and its antagonist, *T. asperellum*, on decayed pineapple.

Materials and methods

Microorganisms

Five *F. guttiforme* (E-261, E-480, E-248, E-384 and E-382) and four *T. asperellum* strains (M25, T33, CBMAI 1364 and Env-01) were used in this study. *Fusarium guttiforme* isolates were obtained from the INCAPER Culture Collection (INCAPER). Most *T. asperellum* strains were obtained from the Brazilian Collection of Environmental and Industrial Microorganisms/University of Campinas (CBMAI). One *T. asperellum* strain, Env-01, was isolated from the environment (air contamination) in Portugal. *Escherichia coli* strain DH5α was obtained from the Micoteca da Universidade do Minho (MUM). All isolates were obtained from preserved cultures at −80 °C. Cryovials were thawed, opened and the strains sub-cultured.

Pathogenicity assays

The Perola cultivar, a standard for susceptibility, was inoculated with the *F. guttiforme* E-261 strain. This fungal strain when grown directly on pineapple (pa) side shoots is hereafter referred to E-261^{pa}. The same strain grown on potato dextrose agar (PDA, Oxoid CM0139) culture plate (cp) is hereafter referred to E-261^{cp}. Finally, the four additional *F. guttiforme* strains, E-480, E-248, E-384 and E-382, were used for comparisons and grown only on PDA.

A 5-mm diameter hole was made in the pineapple side shoots by means of a drill. A 100 μL suspension of *F. guttiforme* strain E-261 in water containing 10^5 conidia mL^{-1} was dispensed into the hole. Inoculated shoots were placed in sterile plastic bags and incubated at 25 °C for 3 days in the dark without humidity control. Samples of side shoots were reserved for the MALDI-TOF analysis. Furthermore, three inoculated samples were sprayed using a microsyringe containing a 6.0 μL water suspension of *T. asperellum* strain Env-01 spores (≈10^5 conidia mL^{-1}). These samples were incubated at aerobic conditions in a 12:12 h light:dark incubator at 25 °C for three days and without humidity control.

MALDI-TOF MS analysis

Different methodologies for spectral acquisition were evaluated based on our previous experience (Oliveira et al. 2015; Silva et al. 2015; Dias et al. 2011; Rodrigues et al. 2011). The optimum methodology leading to reproducible spectra with ion production just above the threshold was achieved as follows. For the *F. guttiforme* strain growing in the pineapple, a pipette tip was used to collect fungal cells by scraping the pineapple that were then spotted onto the 48 well MALDI flex target plate (FlexiMass™, Shimadzu Biotech). The matrix solution (75 mg mL^{-1} 2,5-dihydroxybenzoic acid [DHB] in ethanol/water/acetonitrile [1:1:1] containing 0.03 % (v/v) trifluoroacetic acid [TFA]) was added, gently mixed, and samples were air dried at room temperature. Each pineapple sample was spotted in duplicate to test reproducibility. Cells of *T. asperellum* Env-01^{pa} grown on pineapple, as an antagonist of *F. guttiforme* strain E-261^{pa}, were gently collected from the pineapple/*F. guttiforme* matrix complex. MALDI-TOF sample preparation was performed using DHB matrix solution as described above.

For the spectral analyses of fungal isolates grown on PDA, fungal spores were inoculated on PDA 9-cm plates. Incubation of *T. asperellum* cultures were standardised at aerobically conditions in a 12:12 h light:dark incubator for 3 days at 25 °C. Incubation of *F. guttiforme* cultures were also standardised on PDA plates in the dark for 3 days at 25 °C. Approximately 1 μg of young mycelium and spores of each fungus was transferred directly from the culture plate to the MALDI flex target plate. Immediately, 0.5 μL DHB matrix solution was added and mixed gently.

Escherichia coli strain DH5α was used for *in loco* protein extraction and MALDI-TOF MS calibration as a control. Cells were grown and maintained on Luria-Bertani agar medium (LB: 10 g L^{-1} Bacto-tryptone, 5 g L^{-1} Bacto-yeast extract,

10 g L^{-1} NaCl, 15 g L^{-1} Bacto-agar). *Escherichia coli* DH5α incubation was standardised at 20 h and it was grown aerobically at 37 °C. All cultures were checked for purity prior to use and were subcultured at least once prior to analysis. Approximately 1 μg of cells (about 10^7 cells per sample) was directly transferred from a single colony and spotted onto the 48 well MALDI flex target plate. The matrix solution was added and samples were air dried at room temperature. Each sample was spotted in duplicate to test reproducibility.

During the analyses all solutions were freshly prepared and kept at 5 °C. The analyses were performed on an Axima LNR system (Kratos Analytical, Shimadzu) equipped with a nitrogen laser (337 nm), where the laser intensity was set just above the threshold for ion production. Ions of 12 well-defined ribosomal proteins of intact *E. coli* DH5α cells (4,365.4, 5,096.8, 5,381.4, 6241.4, 6255.4, 6316.2, 6411.6, 6856.1, 7158.8, 7274.5, 7872.1, 9742 and 12227.3 Da) were used as external calibrants. The mass spectra based on the mass range from 2 to 20 kDa were recorded using the linear mode with a delay of 104 ns and an acceleration voltage of +20 kV. The final spectra were generated by summing 20 laser shots accumulated per profile and 50 profiles produced per sample, which led to 1, 000 laser shots per summed spectrum.

Resulting ion peak lists were exported to the Spectral Archive And Microbial Identification System (SARAMIS™) software package where the final microbial identification was achieved. In SARAMIS™, peak lists of individual samples were compared to a database generating a ranked list of matching spectra. For each sample analysed, only those data with match≥99 % spectral similarity were used. This software uses a point system based on ion peak lists with mass signals weighted according to their specificity. The similarity between individual spectra is expressed as the relative or absolute number of matching mass signals after subjecting the data to a single link agglomerative clustering algorithm. Microbial identifications by the SARAMIS™ package are based on the presence or absence of each ion peak in the spectra. A dendrogram of spectral proximity between isolates was created with this software.

Molecular-based identification

Fungal cultures of *F. guttiforme* were grown in complete medium (CM: saccharose 30 g L^{-1}, Na_2NO_3 2 g L^{-1}, protein hydrolysate N-Zamine 2.5 g L^{-1}, yeast extract 5 g L^{-1} and vitamin stock solution 10 mL L^{-1}) (Leslie and Summerell 2006) under 100 rpm in a rotary shaker at room temperature for 12:12 h light:dark conditions for two days. After incubation, the fungal biomass was filtered, the excess water removed, and grounded to a powder with liquid nitrogen using a mortar and a pestle. Then, the DNA was extracted with a standard protocol based on the cetyl trimethyl ammonium bromide (CTAB) technique (Leslie and Summerell 2006). Portions of the translocation elongation factor 1-α (*tef-1*) genes were amplified by PCR from the DNA of the isolates using the primers and amplification conditions of O'Donnell *et al.* (1998). The sequences obtained were aligned with reference sequences of *Fusarium* species belonging to the *Gibberella fujikuroi* species complex and subjected to phylogenetic analysis using maximum parsimony method. *Trichoderma* isolates were previously identified through ITS-rDNA gene region as described by Da Silva *et al.* (2008) and their data are employed herein.

Macro- and micro-morphology

All fungal isolates were submitted to morphological analyses. For each isolate, spores from 7-days-old cultures on PDA were suspended in 500 μL of 0.2 % agar. The suspension was used for single-point inoculation on 9 cm diameter Petri dishes containing 20 mL of PDA and malt extract agar (MEA: Oxoid CM0059). *Fusarium* cultures were incubated for 7 days, in the dark, at 25 °C. On the other hand, *Trichoderma* cultures were incubated for 7 days, in intermittent periods of light and dark, at 25 °C. Then, all isolates were analysed for evaluation of macroscopic and microscopic traits (conidial morphology). Identification followed the taxonomic keys and guides available for *Fusarium* and *Trichoderma* (Leslie and Summerell 2006; Samuels et al. 2011; Ventura 2000).

Results and discussion

The morphological identification of all *Fusarium* isolates was confirmed by molecular analysis using *tef-1* (data not shown). In addition, the morphological identifications of *Trichoderma* isolates MP049, MP094 and MP095 corroborated with the previously identification using ITS region as reported by Da Silva *et al.* (2008). No clear differences were found between the MALDI-TOF MS spectra of *F. guttiforme* E-261^{pa} (pineapple) and E-261^{cp} (agar) (Fig. 1).

The mass spectra for the *F. guttiforme* showed profiles very close to ion peaks ranging from 2 to 11 kDa (Fig. 1). Overall, the number of observed ion peaks was not as large as those observed for other microbial groups (e.g., bacteria) analysis by MALDI-TOF MS, which is expected for the analysis of fungal species by this technique (Passarini et al. 2013; Rodrigues et al. 2014; Santos et al. 2010). A common group of ion peaks was observed within the range 4800 to 4900 Da. These ions were the most intense ones found in the overall MALDI-TOF MS spectra. All strains of *F. guttiforme* showed a common ion peak at 4900 Da. Similarly, in the spectral region within the range from 7200 to 7400 Da a common ion peak at 7325 Da for all strains was observed. In contrast, only the E-480, E-248, E-384 and E-382 strains showed a common ion peak at 7225 Da. Based on the whole spectral

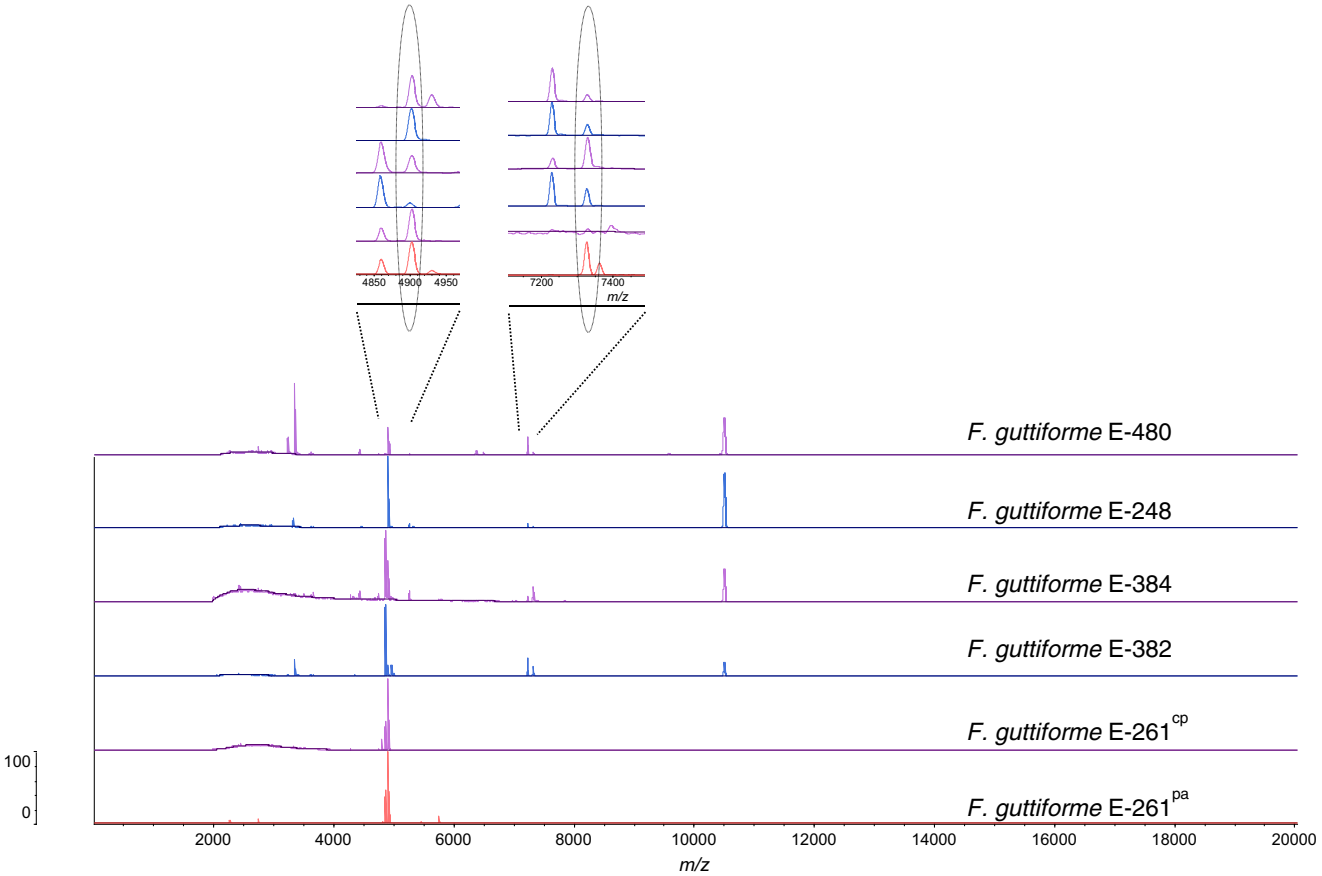

Fig. 1 MALDI-TOF MS spectra of *Fusarium guttiforme* isolates, including E-216^{cp} grown on PDA culture plate and E-216^{pa} grown in pineapple stem side shoot. On the top, the zoom in on two spectral regions to highlight the common ion peaks at 4900 and 7325 Da

data, these four strains are much close related to each other than to E-261 under both growth conditions. This finding is also corroborated by both clustering analysis using SARA MIS™ software (Fig. 2) and by the ITS-rDNA sequencing analysis (data not shown). Hence, E-261 strain is rather different from the other strains. Clustering of the spectral data (Fig. 2) allowed the observation of a small deviation in the percentage of similarity between E-261^{cp} grown on PDA when compared with the strain analysed directly from pineapple side shoot. However, all *F. guttiforme* isolates grouped in the same cluster. Overall, the comparison of the MALDI-TOF MS spectra (Fig. 1) and the clustering analysis (Fig. 2) for *F. guttiforme* strain E-261 grown by the different conditions, suggests that growth conditions did not affect significantly the spectral fingerprint and, consequently, grouping of isolates. The growth conditions were less important than the intra-specific proteomic traits for each isolate allowing the early detection of *F. guttiforme* infecting pineapple cultivar.

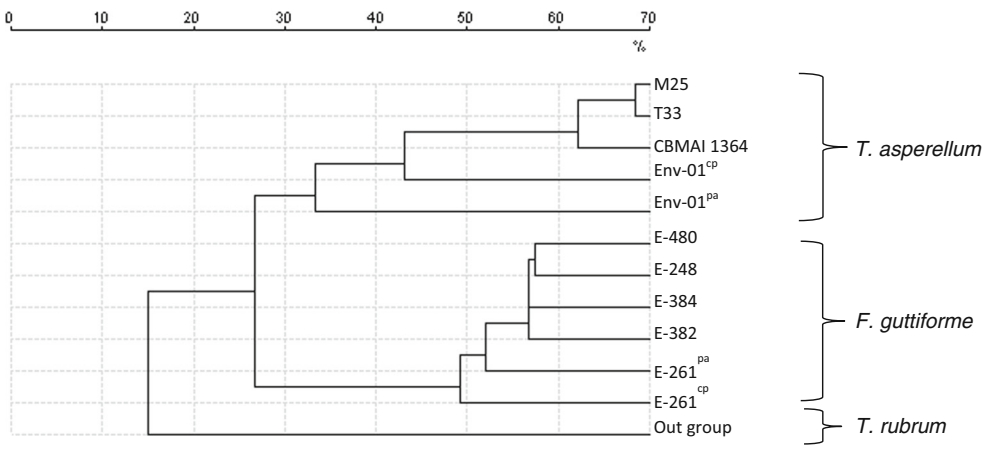

Fig. 2 Clustering MALDI-TOF MS spectral data of *Fusarium guttiforme* and *Trichoderma asperellum* isolates. *Trichophyton rubrum* was used as out group

After direct analysis of *F. guttiforme* by MALDI-TOF MS, the infected pineapple side shoot was then inoculated with an isolate of *Trichoderma asperellum* (Env-01^{pa}). This isolate grown up on the pineapple side shoot acting as an antagonist of *F. guttiforme* E-261^{pa}. The *T. asperellum* E-261^{pa} mycelium/spores mixture was directly analysed by MALDI-TOF MS without any previous treatment. Furthermore, in order to reconfirm its identification, isolate Env-01^{pa} was purified and grown on culture plate (Env-01^{cp}). Then, it was analysed by MALDI-TOF MS and by macro- and micro-morphologies. MALDI-TOF MS spectra of the side stem and purified strains clustered using SARAMIS™ software. Finally, both *T. asperellum* isolates were correctly identified through the MALDI-TOF MS analysis (Fig. 2). Different morphological information characters were taken into consideration for their identification using the hierarchical *Trichoderma* online dichotomous key (Samuels et al. 2011). Morphology corroborated the MALDI-TOF MS data and identified both isolates as *T. asperellum*.

The MALDI-TOF MS data of the two Env-01^{pa} and Env-01^{cp} growth conditions were matched with the spectral data of reference *T. asperellum* strains M25, T33 and CBMAI 1364 (Fig. 3). Both isolates presented similar MALDI-TOF MS profiles. However, the spectral differences found between them (Figs. 2 and 3) were bigger than those found between the equivalent *F. guttiforme* isolates (Figs. 1 and 2). In addition, there was no similarity between the spectra of *F. guttiforme* and *T. asperellum* spectra (Figs. 1 and 3). All *T. asperellum* strains had a set of common ion peaks. M25, T33, CBMAI 1364, Env-01^{pa} from the side stem and Env-01^{cp} grown on PDA had common ion peaks at 7317, 7355 and 7429 Da. Previous studies indicated that 50 to 70 % these ions are derived from ribosomal proteins. The remaining ions are derived from other compounds such as polysaccharides, lipids, phospholipids, and chitin. (Santos et al. 2010)

For strain *T. asperellum* Env-01 grown on the side stem and PDA, differences on the expression of some of these chemical compounds (Fig. 3, top right) can be explained by differential the growth conditions. However, the cluster analysis used to group them is based on the presence or absence of these chemicals. The concentration of each molecule in the sample is not taken into consideration. All of the low intensity peaks are important for species identification (Fig. 3, top left) and clustering. Even either tending to be underestimated or not clearly distinguishing from noise, these peaks are crucial for spectral grouping, as discussed above.

A set of ion peaks in the range 4080 Da to 4510 Da was common for the *T. asperellum* strains M25, T33 and CBMAI

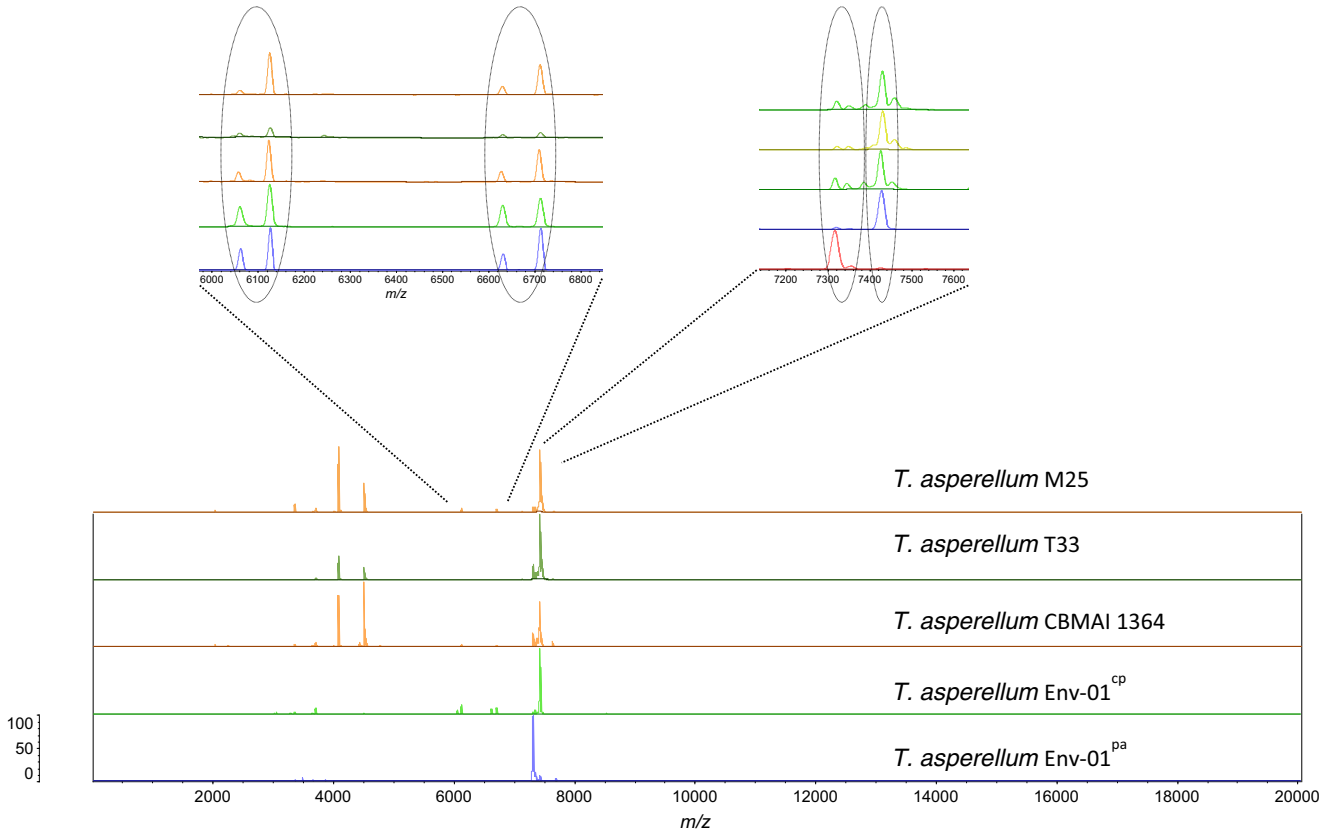

Fig. 3 MALDI-TOF MS spectra of *Trichoderma asperellum* strains M25, T33 and CBMAI 1364, and isolates Env-01^{cp} and Env-01^{pa}. On the top, the zoom in on two spectral regions to highlight the common peaks

1364 which were isolated from the southern Atlantic Ocean marine sponge (Passarini et al. 2013), whereas the *T. asperellum* environmental isolate Env-01 was isolated from the air sample in Portugal.

In summary, the proposed methodology was sensitive and accurate for the detection of *F. guttiforme* infecting pineapple and the identification of *T. asperellum* acting as antagonist against *F. guttiforme* species. The fungal growth conditions seemed less important than the intra-specific peak variation for each *Fusarium* strain. Furthermore, *T. asperellum* was successfully identified when grown jointly with *F. guttiforme* on pineapple side shoots. The spectral differences based on different growth conditions depended on the genus or species analysed. MALDI TOF MS is more cost-effective than other techniques and may be suitable as a high-throughput system.

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