Fungal Biology 123 (2019) 594–600

Contents lists available at ScienceDirect

Fungal Biology

journal homepage: www.elsevier.com/locate/funbio

# Monitoring indole alkaloid production by *Penicillium digitatum* during infection process in citrus by Mass Spectrometry Imaging and molecular networking

Jonas Henrique Costa [a], Jaqueline Moraes Bazioli [a, b], Eder de Vilhena Araújo [a], Pedro Henrique Vendramini [a], Mariana Cristina de Freitas Porto [c], Marcos Nogueira Eberlin [a], Jayme A. Souza-Neto [c], Taícia Pacheco Fill [a, *]

[a] Institute of Chemistry, Universidade Estadual de Campinas, CP 6154, 13083-970, Campinas, SP, Brazil
[b] Faculty of Pharmaceutical Sciences, Universidade Estadual de Campinas, 13083-859 Campinas, SP, Brazil
[c] São Paulo State University (UNESP), School of Agricultural Sciences, Department of Bioprocesses and Biotechnology, Central Multiuser Laboratory, Botucatu, Brazil

ARTICLE INFO

*Article history:*
Received 15 January 2019
Received in revised form
26 February 2019
Accepted 6 March 2019
Available online 22 March 2019

Corresponding Editor: Gustavo Henrique Goldman

*Keywords:*
Alkaloids
Citrus green mold
Insecticidal activity
Secondary metabolites

ABSTRACT

Green mold, caused by *Penicillium digitatum*, is the most destructive post-harvest disease in citrus. Secondary metabolites produced by fungal phytopathogens have been associated with toxicity to their respective host through the interaction with a wide range of cell targets. Natural products have also been described as important molecules for biocontrol and competition in their respective environment. For *P. digitatum*, the production of indole alkaloids, tryptoquialanines A and B, have been reported. However, their biological role remains unknown. Mass Spectrometry Imaging (MSI) technique was applied here for the first time to monitor the secondary metabolites produced on the orange surface during infection in order to gain insights about the *P. digitatum*-citrus interaction mechanisms. Through the combination of MSI and molecular networking it was possible to report, for the first time, the production of tryptoquivalines and fumiquinazolines by *P. digitatum* and also the accumulation of tryptoquialanines on the fruit surface from 4 to 7 d post inoculation. *P. digitatum* was also evaluated concerning the ability to sinthesize indole alkaloids *in vivo* in the different citrus hosts. The biological role of tryptoquialanines was investigated and tryptoquialanine A was submitted to insecticidal bioassays that revealed its high toxicity against Aedes Aegypti, suggesting an important insecticidal action during orange decay.


## 1. Introduction

*Penicillium digitatum* is the most severe postharvest pathogen of citrus fruits and has been reported to cause the green mold disease, contributing to up to 90 % of the total post harvest losses in tropical sub-climates (Ghooshkhaneh et al., 2018; Macarisin et al., 2007). *P. digitatum* is able to infect citrus fruits through pores and wounds caused by environmental factors, or during harvest process and transport (Perez et al., 2017).

There are several factors that mediate the host–pathogen interaction between *P. digitatum* and citrus fruit, including hydrogen peroxide modulation and secretion of organic acids by the phytopathogen (Macarisin et al., 2007), resulting in pH control, leading to an optimal pH for specific cell wall degrading enzymes, such as polygalacturonases (PG) (Barmore and Brown, 1981; Prusky et al., 2004; Zhang et al., 2013). Nevertheless, secondary metabolites directly associated with the infection process *in situ* have not been identified so far (Zhu et al., 2017).

Ariza et al. (2002) reported indole alkaloids, such as tryptoquialanines A and B, as secondary metabolites of *P. digitatum*, which are biosynthesized on citrus fruits during infection. The authors also reported that the fungal infection on natural substrates induced the production of citrus monoterpenes together with fungal volatiles, such as sesquiterpenes. However, molecular studies conduced by Zhu et al. (2017) indicated that tryptoquialanines are not related to *P. digitatum's* pathogenicity in citrus fruits. The deletion of *tqaA* gene (nonribossomal peptide synthetase) did not affect the virulence of the gene knockout mutant when

* Corresponding author.
*E-mail address:* taicia@unicamp.br (T.P. Fill).

https://doi.org/10.1016/j.funbio.2019.03.002
1878-6146/

compared to the wild type strain, so the exact biological role of tryptoquialanines is still unknown (Zhu et al., 2017).

The studies concerning this pathogen-host system mainly focus on the fruit response to the pathogen or on the fungicide resistance (Lopéz-Peréz et al., 2015), so the pathogenicity mechanisms and infection process are still unclear (Zhu et al., 2017). Therefore, it become necessary to seek a better understanding of these mechanisms in different citrus fruits, in order to discover secondary metabolites potentially associated with the citrus infection. Understanding the infection process and the fungus strategies is an important step to develop ways to protect citrus from *P. digitatum* infections leading to a more productive citriculture worldwide.

In this context, Mass Spectrometry Imaging (MSI) is a powerful and useful tool to describe the functional roles of secondary metabolites in a biological context, based on their relative abundances of *m/z* and spatial distribution (Lei et al., 2011). Through MSI it is possible to investigate and identify secondary metabolites involved at different stages of a phytopathogen infection. Here we explored different *P. digitatum* infection stages by Mass Spectrometry Imaging combined with Molecular Networking using GNPS approach, to profile the secondary metabolite production of *P. digitatum* during infection process. The biological role of indole alkaloids during this process is also discussed.

## 2. Materials and methods

### 2.1. Fungal culture

The *P. digitatum* strain used in the studies is deposited with the Spanish Type Culture Collection (CECT) under the accession code CECT20796. *P. digitatum* was maintained on commercial potato dextrose agar (PDA) (Acumedia). PDA was autoclaved at 103 KPa (121 °C) for 15 min. PDA plates were stored at 25 °C for 7 d in darkness. Spores were harvested by washing the agar surface with sterile distilled water and subsequently diluted to a final concentration of $10^6$ spore $mL^{-1}$.

### 2.2. Mass spectrometry imaging (MSI)

MSI analysis were performed directly on the orange (*Citrus sinensis*) peel surface infected with *P. digitatum* using a Prosolia DESI source Modelo Omni Spray 2D®-3201) coupled to a Thermo Scientific QExactive® Hybrid Quadrupole-Orbitrap Mass Spectrometer. The DESI configuration used was the same set by Angolini et al. (2015) with small modifications. The methanol flow rate was set at (10.0 μL $min^{-1}$). MS data was processed with Xcalibur software (version 3.0.63) developed by Thermo Fisher Scientific. The DESI-MSI data was converted into image files using Firefly data conversion software (version 2.1.05) and viewed using BioMap software (version 3.8.0.4) developed by Novartis Institutes for BioMedical Research. In BioMap, color scaling was adjusted to a fixed value for comparison between the samples.

### 2.3. Large scale experiment for isolation of compounds **1**, **3** and **7**

Large scale cultivation was performed by cultivating *P. digitatum* in 12 L of PD (Potato-Dextrose) liquid media in sterile 1 L Erlenmeyer and stored at 25 °C for 10 d in darkness. After incubation, culture broth was extracted twice with equal volumes of ethyl acetate 100 % (1:1 v/v) and vacuum filtered. Solvent was removed under reduced pressure and the final extract stored at −20 °C.

### 2.4. Isolation of secondary metabolites **1**, **3** and **7**

High performance liquid chromatography (HPLC) separations for **1**, **3** and **7** were performed on a Phenomenex column Luna 5 μm Phenyl-Hexyl (250 × 4.6 mm) using a SHIMADZU prominence HPLC LC-20AT, equipped with CBM-20A communication bus module, SPD-M20A photodiode array detector and SIL-20A auto sampler. The mobile phase was water (A) and acetonitrile with 0.1 % (v/v) of formic acid (B). Flow rate was 1.0 mL $min^{-1}$. The eluent profile (A:B) was: 0–50 min, gradient from 65:35 to 50:50; 50–70 min, gradient from 50:50 to 40:60. Preparative HPLC purifications for **1**, **3** and **7** were performed on a Phenomenex column Luna 5 μm Phenyl-Hexyl (250 × 10 mm) using a Waters 1525 Binary HPLC Pump equipped with Waters 2998 Photodiode Array Detector and Waters Fraction Collector III using the same optimized gradient conditions with a flow rate set at 4.7 mL $min^{-1}$.

### 2.5. Characterization of secondary metabolites **1**, **3** and **7**

$^1$H NMR, $^{13}$C NMR and 2D experiments were acquired in a Bruker Avance III 500 ($^1$H 500.13 MHz and $^{13}$C 125.7 MHz). Deuterated chloroform ($CDCl_3$; 7.23 ppm), dimethyl dulfoxide (DMSO; 2.50 ppm and 39.51 ppm) and tetramethylsilane (TMS; 0.0 ppm) were used as a solvent and internal reference. Chemical shifts (δ) were expressed in (ppm) and the coupling constants (J) in Hertz (Hz).

### 2.6. In vivo *and* in vitro *assays*

For *in vivo* assays, mature oranges (*Citrus sinensis*), sicilian lemons (*Citrus limon*) and tangerine (*Citrus reticulata*) obtained from a local grocery store (Campinas, SP, Brazil) were surface-sterilized with 2 % (v/v) sodium hypochlorite solution (Panebianco et al., 2014), rinsed with distilled water and air-dried at room temperature. The fruits were wounded at the equatorial region (1 cm wide x 1 cm deep) and 15 μL of *P. digitatum* spore solution was inoculated in the wound site. Infected and control fruits were stored in sterile 500 mL beakers. For *in vitro* assays, 15 μL of spore solution were inoculated in 100 mL of sterile orange juice or in 20 mL of PDA. All assays were done in duplicate and stored at 25 °C for 7 d in darkness. After incubation time, the fruits peel were cut (2 cm × 2 cm) and the extractions of the secondary metabolites produced in the fruits were made with 5 mL ethyl acetate 100 % during 1 h in ultrasonic bath. Orange juice and PDA were extracted twice with equal volumes of ethyl acetate 100 % (1:1 v/v) during 1 h in ultrasonic bath. All the extracts were filtered, dried under $N_2$ and stored at −20 °C.

### 2.7. MS/MS analysis

Crude extracts were resuspended in MeOH HPLC and centrifuged at 13,000 rpm for 5 min. The samples were analyzed using a LC Agilent 1200 mass spectrometer coupled with Agilent iFunnel 6550 Q-ToF LC-MS. The electrospray ionization source operated in positive mode ESI (+), following operating conditions: nebulizing gas temperature: 290 °C; capillary voltage: +3500 V; nozzle voltage: 320 V; drying gas flow: 12 mL $min^{-1}$; nebulization gas pressure: 50 psi; auxiliary gas temperature: 350 °C and flow of auxiliary gas: 12 mL $min^{-1}$. The analyzer time of flight (ToF) operated in the range *m/z* 50–1500. Collision Energy formula (auto MS/MS mode): 4 V (slope)*(*m/z*)/100 + 5 V (offset). Maximum 5 precursors per cycle were selected. 2 μL of sample were injected. Stationary phase: Thermo Scientific column Accucore C18 2.6 μm, 2.1 mm × 100 mm. The mobile phase was water (A) and acetonitrile with 0.1 % (v/v) of formic acid (B). Flow rate was 0.2 mL $min^{-1}$. The

eluent profile (A:B) was: 0–10 min, gradient from 95:5 to 2:98; 10–15 min, isocratic elution with 2:98; 15–16.2 min, gradient from 2:98 to 95:5; 16.2–20 min, isocratic elution with 95:5. The spectra were processed with Agilent Mass Hunter Workstation Software.

### 2.8. Molecular MS/MS network

A molecular network for *P. digitatum* cultivated in sicilian lemon, tangerine, orange, orange juice and PDA was created using the online workflow at GNPS (http://gnps.ucsd.edu). The data was filtered by removing all MS/MS peaks within ±17 Da of the precursor *m/z*. MS/MS spectra were window filtered by choosing only the top 6 peaks in the ± 50 Da window throughout the spectrum. The data was then clustered with MS-Cluster with a parent mass tolerance of 0.2 Da and a MS/MS fragment ion tolerance of 0.1 Da to create consensus spectra. Further, consensus spectra that contained less than 2 spectra were discarded. A network was then created where edges were filtered to have a cosine score above 0.7 and more than 6 matched peaks. Further edges between two nodes were kept in the network only if each of the nodes appeared in each other's respective top 10 most similar nodes. The spectra in the network were then searched against GNPS′ spectral libraries. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.7 and at least 6 matched peaks.

### 2.9. Evaluation of insecticidal activity of tryptoquialanine A

Larvae for all experiments were 2nd instars and kept under controlled temperature conditions (T = 27 ± 1 °C), relative humidity (RH = 70 ± 5 %) and photoperiod of 12 h. The bioassay was performed following the criteria established by Dulmage et al. (1990), with some modifications. Ten *Ae. aegypti* larvae were placed in individual wells of a 6-well cell culture dish containing 10 mL water, 100 μL of liquid feed and the application of extract to the concentration of 250 μg/mL was added to the larval water; the negative control receiving only 1 % DMSO (Dimethylsulfoxide) at the same concentrations, whose mortality did not exceed 10 %. Survival was monitored for four days checking for the number of live and dead larvae of each well. Three independent biological replicates (with three technical replicates each) where performed for each experiment.

## 3. Results and discussion

### 3.1. MSI analysis of green mold on orange fruits surface

MSI has become an important tool for mycologists since it is able to image thousands of molecules, including metabolites, proteins, lipids and peptides, providing a visualization of the spatial distribution of secondary metabolites on fungal cultures (Sica et al., 2014; Buchberger et al., 2018). A few *P. digitatum* secondary metabolites are described, however, their biological roles in infection have not yet been fully unveiled (Zhu et al., 2017) as the pathogenicity mechanisms and host specificity (Marcet-Houben et al., 2012). MSI technique was applied here for the first time to investigate the metabolites produced by *P. digitatum* on citrus fruits surface during infection process and how they are distributed on the infected fruits, giving initial insights into the role of secondary metabolites in the infection mechanisms of *P. digitatum* in the host.

DESI-MSI was applied on oranges infected with *P. digitatum* at different stages of the infection process (from 4 to 7 dpi) and an orange control (Fig. 1). It was possible to detect, in all infection stages, the tryptoquialanines A and B and their biosynthetic intermediates. Fig. 1 shows the MSI signals obtained for ions $[M+H]^+$ at *m/z* 519.1870, *m/z* 505.1712, *m/z* 475.1612, *m/z* 460.1976 and *m/z* 459.1664 which correspond respectively to tryptoquialanine A (**1**) ($C_{27}H_{26}N_4O_7$), tryptoquialanine B (**2**) ($C_{26}H_{24}N_4O_7$), which are already described to be produced by *P. digitatum* (Ariza et al., 2002), tryptoquialanone (**4**) ($C_{25}H_{22}N_4O_6$), 15-dimethyl-2-epi-fumiquinazoline A (**5**) ($C_{25}H_{25}N_5O_4$), and deoxytryptoquialanone (**6**) ($C_{25}H_{22}N_4O_5$) that are intermediates of the tryptoquialanine biosynthetic pathway. Tryptoquialanines biosynthesis pathway was well unveiled for *Penicillium aethiopicum* by Gao et al. (2011) and intermediates **4**, **5** and **6** were also detected for *P. digitatum* through MSI analysis, since tryptoquialanine biosynthetic gene clusters of both fungi show high similarity (Zhu et al., 2017) as show in Supplementary Fig. S13. All structures (Fig. 2) have been confirmed through exact masses and typical fragmentation pattern of tryptoquialanine-like alkaloids. In MS/MS analysis, main typical fragments were observed at $[M+H]^+$ *m/z* 156.07, *m/z* 197.10 and *m/z* 213.10.

In Fig. 1 it is observed that the alkaloids signals are only present on the infected surface, indicating that these compounds are produced during the mycelial growth of *P. digitatum*. It is also noticed an accumulation of the metabolites on the fruit surface: the intensity of the signals increases in relation to the time of infection, especially at 6 dpi, where there is higher concentration level of these alkaloids in comparison to the previous stages of infection. Zhu et al. (2017) confirmed through RNA-seq analysis that the expression of the tryptoquialanine biosynthetic genes occurs since the first day after inoculation and are in highest level after three days, indicating a potential biological role of these compounds. Fig. 1 also shows that the ion *m/z* 460.1973 relative to the intermediate **5** presents a different spatial distribution compared to the other analyzed alkaloids being mainly found in the peripheral zone (white areas). It suggests that **5** is one of the initial intermediates in the biosynthetic route of tryptoquialanine A (Gao et al., 2011), and is in higher concentration in the youngest fungal cells.

The detection of the known tryptoquialanines A and B through MSI shows that this technique can be used for searching new secondary metabolites. MSI analysis also revealed ions ($[M+H^+]$ *m/z* 433.1502 and *m/z* 503.1920) never before reported from *P. digitatum*. These compounds were isolated and investigated by NMR spectroscopy, since they are not part of the described tryptoquialanine biosynthetic pathway.

### 3.2. Isolation and characterization of secondary metabolites *1*, *3* and *7*

Secondary metabolites are small organic molecules produced by various microorganisms and some of them have been shown to contribute to the pathogenicity of several pathogenic fungi (Scharf et al., 2014). The discovery of new secondary metabolites produced by *P. digitatum* and the understanding of their biological role provide more information about the infection process and how this fungus may survive the defense mechanisms of fruit. *P. digitatum* extract was obtained from a scaled up experiment and metabolites of interest were isolated by preparative HPLC. Compounds **3** (*m/z* 503.1920) and **7** (*m/z* 433.1503) were detected first by our MSI technique and were isolated for structural characterization.

Compound **3,** previously isolated by Gao et al. (2011), is not part of the normal biosynthetic pathway of tryptoquialanine A, since it is a final product formed after the deletion of the TaqE enzyme responsible for catalyzing a *N*-hydroxylation and is not yet described as a natural product. Gao et al. (2011) suggested that the remaining tailoring steps in the *tqa* pathway can function in the absence of *N*-hydroxylation, leading to **3**. So, the detection of **3** in the wild-type *P. digitatum* through MSI analysis warranted more investigation. Compound **3**, named as Tryptoquialanine C, was

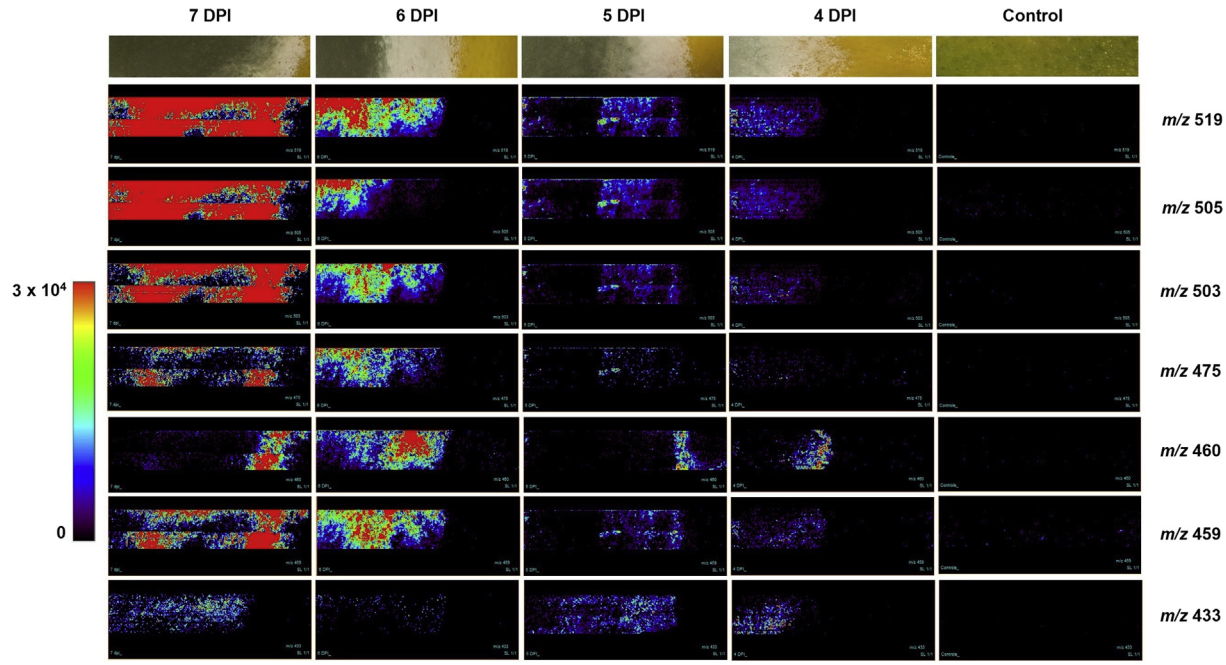


**Fig. 1.** (+) DESI-MSI showing different spacial distributions of molecules over orange peel infected with *Penicillium digitatum* after 4, 5, 6 and 7 d post inoculation (dpi). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

isolated from *P. digitatum* extract and its structure was confirmed by NMR spectroscopy, confirming that it was a new secondary metabolite produced by *P. digitatum*. Supplementary Table S1 shows NMR data obtained for **3**. The NMR signals for **3** are similar to those for **1** (Ariza et al., 2002), with exception of the presence of an NH signal at 3.05 ppm and the absence of an NOH signal ($\delta_H$ 7.95) (Supplementary Fig. S12). $^1$H NMR spectrum presented three singlets and one doublet of methyl groups, one methylene group, three methine groups at $\delta_H$ 5.30 (H-2), 5.40 (H-12) and 6.25 (H-27), and signals corresponding to nine aromatic protons between $\delta_H$ 7.30 and 8.40. Signals characteristic of an imine at $\delta_c$ 153.2 (C-26) and of one amide group at $\delta_c$ 161.7 (C-18) suggested that the other aromatic ring was derived from a part bound to a quinazoline structure. The bonds between the quinazoline

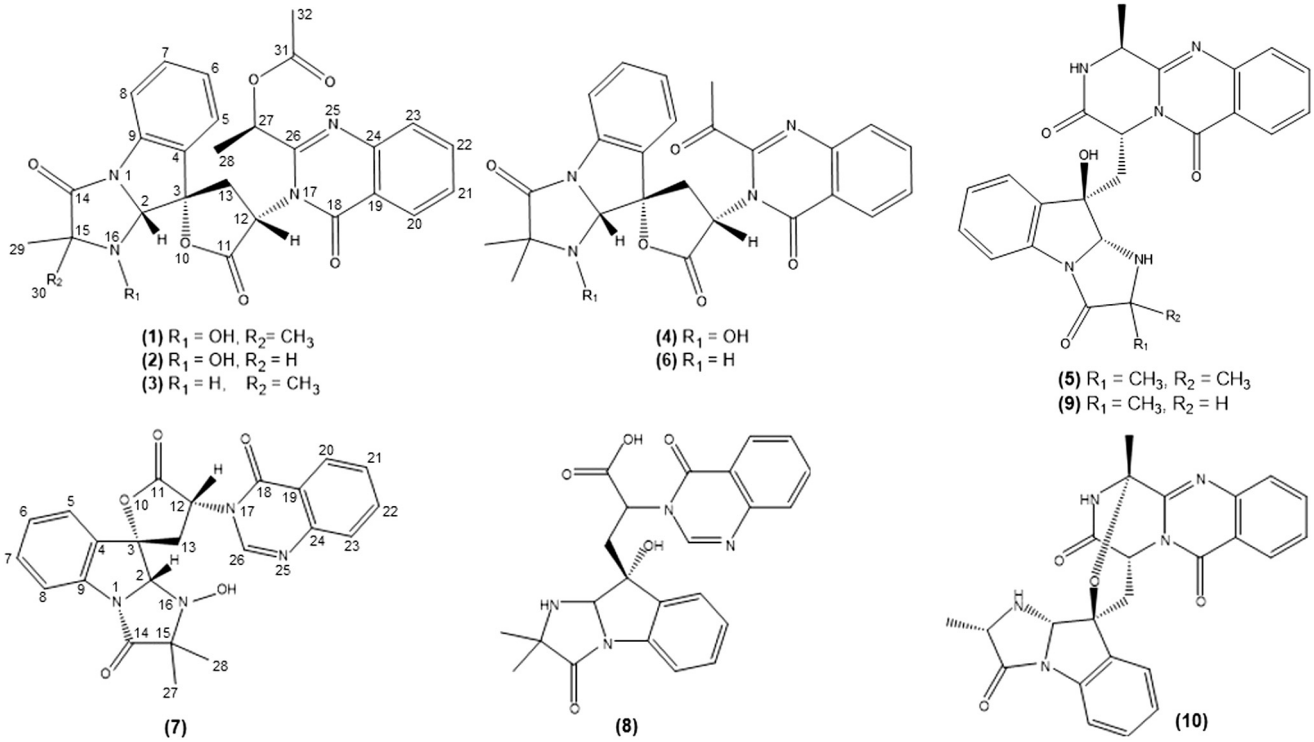


**Fig. 2.** Structures of **1**–**6** detected through MSI analysis and of **7**–**10** identified through GNPS MS/MS database.

structure and the rest of the molecule structure can inferred by HMBC correlations from $\delta_c$ 153.2 (C-26) to $\delta_H$ 6.25 (H-27) and from $\delta_c$ 161.7 (C-18) to $\delta_H$ 5.40 (H-12). Long-range HMBC correlations were also observed from $\delta_c$ 133.9 (C-4) to $\delta_H$ 3.05 (N—H) and from a lactone carbonyl group at $\delta_c$ 162.5 (C-11) to the singlet at $\delta_H$ 5.30 (H-2) (Ariza et al., 2002), allowing elucidation of **3**.

Through $^1$H and $^{13}$C NMR data (Supplementary Table S2) for **7**, it was possible to conclude that compound **7** is tryptoquivaline L, alkaloid first isolated from *Aspergillus fumigatus* and well characterized in the literature (Yamazaki et al., 1979) (Supplementary Fig. S16 ans S17). Tryptoquivaline L has never have been reported, until now, as a *P. digitatum* metabolite in the literature.

Compound **1** was also isolated and its structure was confirmed through $^1$H and $^{13}$C NMR data (Supplementary Figs. S19 and S20). The NMR signals obtained for **1** were in agreement with the data already reported in the literature for this compound (Ariza et al., 2002).

### 3.3. Evaluation of insecticidal activity of tryptoquialanine A

Zhu et al. (2017) evaluated the involvement of tryptoquialanines produced *P. digitatum* in the citrus infection process. The authors deleted the *tqaA* gene (Non-ribosomal peptide synthetase) responsible for tryptoquialanine A production. Phenotype assays suggested no significant variation between the non-TQA mutant and the wild-type strain in fungal growth, concluding that tryptoquialanine A was not involved in the pathogenicity of *P. digitatum* and does not act as a virulence factor, implying a different biological role for the indole alkaloids (Zhu et al., 2017).

Based on structure similarity, tryptoquialanine A (**1**) is considered a tremorgenic mycotoxin (Ariza et al., 2002) acting on the central nervous system of vertebrate animals, causing symptoms such as decreased activity and immobility (Gao et al., 2011). Since **1** is not related to the *P. digitatum* pathogenicity, Zhu et al. (2017) suggests that it may have an insecticidal biological function within its micro ecosystem.

Compound **1** was isolated and applied in insecticidal assays to evaluate the hypothesis suggested by Zhu et al. (2017). In 24 h after the exposure to **1**, the mortality rate of *Ae. Aegypti* larvae was 37 %. The larvae mortality gradually increased during the days and reached 81 % on the fourth day (Fig. 3). This result indicates a very pronounced insecticidal activity of **1** and suggests that **1** may be important as a biocontrol against insects during orange decay.

### 3.4. Comparing alkaloids in different citrus: molecular network

Although DESI-MSI is a great tool to discover new secondary metabolites involved in biological interactions, the technique is limited to the analysis of the fruits surface, so that internal metabolites remain undetected (Sica et al., 2014). Other complementary approaches are needed to further characterize the metabolic profile during infection. Therefore, molecular networking was used here, as a complement to DESI-IMS, to annotate secondary metabolites produced during *P. digitatum's* infection.

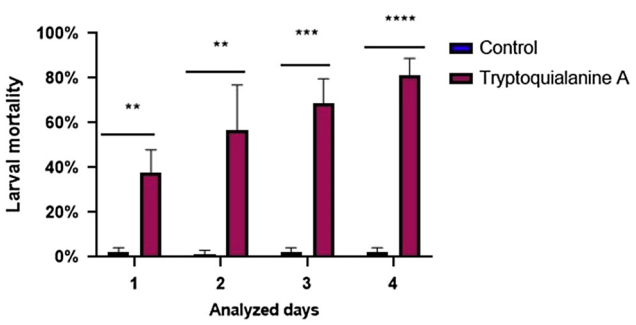


**Fig. 3.** Mean and standard deviation of the mortality rate of *Aedes aegypti* larvae for 96 h. The differences between Tryptoquialanine A treated samples and PBS control samples were evaluated by unpaired t test (**, $p < 0.05$, ***$p < 0.05$, ****$p < 0.0001$), which had significant effects on mortality.

Another alternative to identify unknown compounds, involves metabolite fragmentation patterns acquired through tandem MS analysis, that can be matched to those presented in databases such as GNPS, PubChem and others useful databases for dereplication (Covington et al., 2017; Smith et al., 2005). Nowadays, the largest natural product public database with MS/MS spectra is GNPS: Global Natural Products Social Molecular Networking, containing more than 140.000 natural products (Covington et al., 2017; Gaudêncio and Pereira, 2015).

In this paper, we further investigated the metabolic profile of *P. digitatum* during infection in different citrus hosts. As mentioned before, this fungus is considered a major pathogen of citrus fruit (Ghooshkhaneh et al., 2018). The metabolic profiles of *P. digitatum* in distinct citrus fruits (sicilian lemon, tangerine and orange), orange juice and PDA were analyzed in the GNPS database through tandem MS (MS/MS) aimed at finding new metabolites.

After generating the molecular networks, the node connectivity was visualized (Supplementary Fig. S22). Each node represents one MS/MS spectrum and is labeled with the parent (precursor) mass. Since fragmentation spectra generally reflect the chemical structures of the fragmented ions, it becomes possible to represent the produced metabolites in clusters of similar structures through the GNPS platform (Nguyen et al., 2013) (as observed in Fig. 4). For *P. digitatum* extracts the network contained 31 different clusters.

The visualization of cluster B showed that the ions $[M+H]^+$ *m/z* 519.1871 and 505.1712, clustered together in the molecular network (Fig. 4), are tryptoquialanine A **1** and tryptoquialanine B **2** respectively, major secondary metabolites for *P. digitatum* (Ariza et al., 2002), and observed in all growth media. Interestingly, **1** and **2** were observed *in vivo* in all citrus fruits, suggesting an important biological role, which was confirmed here through the insecticidal biossays.

The tryptoquialanine intermediates detected by MSI analysis were also observed in *P. digitatum* extracts. **5** (*m/z* 460.1976) is one of the precursors of tryptoquialanine pathway and was observed in orange and PDA media. Similarly, **6** (*m/z* 459.1664) and **3** (*m/z* 503.1920) were also observed, being the last one found in all growth media and **6** was only produced in sicilian lemon and PDA media. Finally, **4** (*m/z* 475.1612), another intermediate involved in the tryptoquialanine biosynthesis, was only detected in PDA under the cultivation conditions tested.

In addition, cluster A presented some well-known compounds in the GNPS database bolded by red (Fig. 4). These compounds were identified as tryptoquivaline L **(7)**, tryptoquivaline Q **(8)**, fumiquinazoline A **(9)** and fumiquinazoline C **(10)**, with exact mass of $[M+H]^+$ *m/z* 433.1503, $[M\text{-}H_2O+H]^+$ *m/z* 417.1555 $[M\text{-}H_2O+H]^+$ *m/z* 428.1717 and $[M+H]^+$ *m/z* 444.1665, respectively. All structures are represented in Fig. 2 and are secondary metabolites for the first time described in *P. digitatum* further confirming molecular networking as a great tool for natural products discovery.

Tryptoquivaline L **(7)** was detected in PDA media by GNPS in agreement with the MSI analyses. Both techniques were able to identify **7** as a secondary metabolite produced by *P. digitatum*, and these results were confirmed by the isolation and structure elucidation of **7**. Tryptoquivaline L has been previously reported for the fungus *A. fumigatus* (Yamazaki et al., 1978, 1979), and *Neosartorya siamenisis* KUFC 6349 (Buttachon et al., 2012). This compound was also isolated from marine sponge associated fungus *Neosartorya paulistensis* KUFC 7897 (Gomes et al., 2014) and the marine-derived

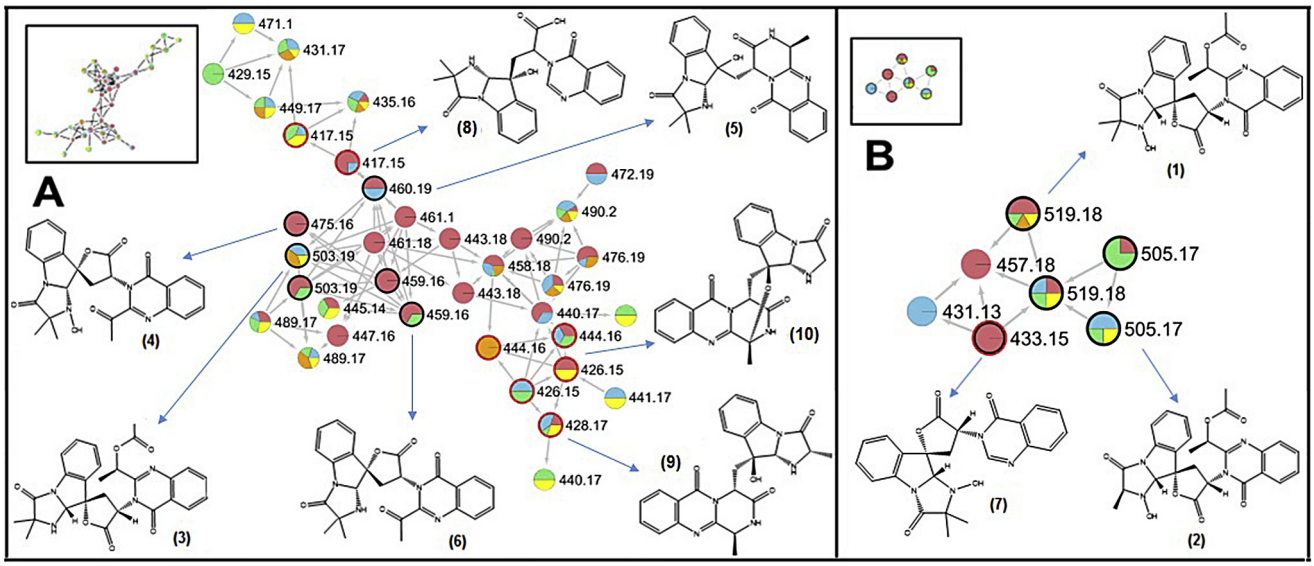


**Fig. 4.** Zoom in of MS/MS network analysis (Supplementary Fig. S22) of PDA, Orange, Sicilian Lemon, Orange juice and Tangerine extracts from *P. digitatum*. Colors indicated in the key correspond to the different growth media source. Nodes bolded by black lines represents the compounds observed in the MSI analysis and nodes bolded by red represents GNPS hits. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

fungus *Neosartorya laciniosa* KUFC 7896 (Eamvijarn et al., 2013), as well as *Neosartorya takakii* KUFC 7898 (Zin et al., 2015). Tryptoquivaline Q **(8)** has been previously isolated from a marine-derived fungus *Neosartorya* sp. HN-M-3 (Sun et al., 2012). Regarding the fumiquinazolines A **(9)** and C **(10)**, Numata et al., (1992) isolated both molecules from the mycelium of *A. fumigatus*. These compounds exhibited moderate cytotoxicity against the cultured P-388 lymphocytic leukemia cells (Numata et al., 1992). Fumiquinazolines and tryptoquialanines present similar enzymes in their biosynthetic pathway and also have Fumiquinazoline F as an intermediate in common (Gao et al., 2011; Ames et al., 2011). Sequence analysis of TqaA enzyme from *P. aethiopicum* tryptoquialanine pathway revealed high homology with Af12080 enzyme from *A. fumigatus* fumiquinazoline pathway. TqAa enzyme, in the tryptoquialanine pathway, catalyzes the biosynthesis of the Fumiquinazoline F, intermediate that can be used in both pathways (Gao et al., 2011). Therefore, is not surprising to detect fumiquinazolines in *P. digitatum*'s extract when we analyze both biosynthetic pathways. Until now, **8, 9** and **10** have never been reported as *P. digitatum* secondary metabolites.

GNPS molecular networking allowed consistent identification of metabolites present not only on the surface of the fruit but also in the different *P. digitatum* extracts. The detection of ions through MSI and GNPS analyses confirm the production of known and new indole alkaloids in the mycelium of *P. digitatum*.

## 4. Conclusions

Despite great economic interest related to *P. digitatum*, studies concerning this pathogen-host system have mainly focused on treatments against infection symptoms. The specificity concerning the host as well as the biological roles of secondary metabolites involved in the pathogen-host interaction remain largely unknown. In this context, MSI has emerged as a powerful tool to gain more insights concerning the natural products produced in *P. digitatum* infection that may have an important biological role. The MSI analysis enabled monitoring and understanding the indole alkaloid production by *P. digitatum* during infection process in citrus. Complementarily to MSI, GNPS molecular networking allowed the study of the metabolic profile produced by *P. digitatum* in different citrus hosts, confirming the production of known and new alkaloids in the mycelium of *P. digitatum*. Therefore, MSI and Molecular Networking taken together, provide a solid insight into different nature interactions and pathogen infection process. Through these complementary approaches it was possible to provide the first insights about the indole alkaloids involved in the *P. digitatum*-citrus interaction and initial thoughts of the possible biological role associated to them. In addition, the knowledge concerning the potential biological role of tryptoquialanines can serve as a target to the development of new biological insecticides to be potentially used in agriculture. In conclusion, the secondary metabolism of *P. digitatum* and the roles of natural products in the infection are still an open field for new research opportunities.

## Acknowledgements

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001, Fundação de Amparo a Pesquisa no Estado de São Paulo [grant number FAPESP 2017/24462-4 and 2018/03670-0] and Pró-Reitoria de Pesquisa Unicamp (PRP/FAEPEX). MCFP is recipient of a PhD Fellowship from CAPES (grant number 8887.137194/2017-00). JASN is recipient of a FAPESP Young Investigator Award (grant number 2013/11343-6).

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2019.03.002.

## References

Ames, B.D., Haynes, S.W., Gao, X., Evans, B.S., Kelleher, N.L., Tang, Y., Walsh, C.T., 2011. Complexity generation in fungal peptidyl alkaloid biosynthesis: oxidation of fumiquinazoline a to the heptacyclic hemiaminal fumiquinazoline C by the flavoenzyme Af12070 from *Aspergillus fumigatus*. Biochemistry 50 (4), 8756-8787-69. http://doi.org/10.1021/bi201302w.

Angolini, C.F.F., Vendramini, P.H., Araújo, F.D.S., Araújo, W.L., Augusti, R., Eberlin, M.N., De Oliveira, L.G., 2015. Direct protocol for ambient mass

spectrometry imaging on agar culture. Anal. Chem. 87, 6925–6930. https://doi.org/10.1021/acs.analchem.5b01538.
Ariza, M.R., Larsen, T.O., Petersen, B.O., Duus, J.Ø., Barrero, A.F., 2002. Penicillium digitatum metabolites on synthetic media and citrus fruits. J. Agric. Food Chem. 50, 6361–6365. https://doi.org/10.1021/jf020398d.
Barmore, C.R., Brown, G.E., 1981. Polygalacturonase from citrus fruit infected with *Penicillium italicum*. Phytopathology 71, 328–331.
Buchberger, A.R., DeLaney, K., Johnson, K., Li, L., 2018. Mass spectrometry imaging: a review of emerging advancements and future insights. Anal. Chem. 90, 240–265. http://doi.org/10.1021/acs.analchem.7b04733.
Buttachon, S., Chandrapatya, A., Manoch, L., Silva, A., Gales, L., Bruyère, C., Kijjoa, A., 2012. Sartorymensin, a new indole alkaloid, and new analogues of tryptoquivaline and fiscalins produced by Neosartorya siamensis (KUFC 6349). Tetrahedron 68, 3253–3262.
Covington, B.C., McLean, J.A., Bachmann, B.O., 2017. Comparative mass spectrometry-based metabolomics strategies for the investigation of microbial secondary metabolites. Nat. Prod. Rep. 34, 6–24. https://doi.org/10.1039/C6NP00048G.
Dulmage, H.T., Yousten, A.A., Singer, S., Lacey, L.A., 1990. Guidelines for Production of *Bacillus Thuringiensis* H-14 and *Bacillus Sphaericus*. UNDP/World Bank/WHO, Steering Committee to Biological Control of Vetores, Geneva, p. 59.
Eamvijarn, A., Gomes, N.M., Dethoup, T., Buaruang, J., Manoch, L., Silva, A., Pedro, M., Marini, I., Roussis, V., Kijjoa, A., 2013. Bioactive meroditerpenes and indole alkaloids from the soil fungus Neosartorya fischeri (KUFC 6344), and the marine-derived fungi Neosartorya laciniosa (KUFC 7896) and Neosartorya tsunodae (KUFC 9213). Tetrahedron 69, 8583–8591.
Gao, X., Chooi, Y.H., Ames, B.D., Wang, P., Walsh, C.T., Tang, Y., 2011. Fungal indole alkaloid biosynthesis: genetic and biochemical investigation of the tryptoquialanine pathway in Penicillium aethiopicum. J. Am. Chem. Soc. 133 (8), 2729–2741. https://doi.org/10.1021/ja1101085.
Gaudêncio, S.P., Pereira, F., 2015. Dereplication: racing to speed up the natural products discovery process. Nat. Prod. Rep. 32, 779–810.
Gomes, N.M., Bessa, L.J., Buttachon, S., Costa, P.M., Buaruang, J., Dethoup, T., Silva, A., Kijjoa, A., 2014. Antibacterial and antibiofilm activities of tryptoquivalines and meroditerpenes isolated from the marine-derived fungi Neosartorya paulistensis, N. laciniosa, N. tsunodae, and the soil fungi N. fischeri and N. siamensis. Mar. Drugs 12, 822–839.
Ghooshkhaneh, N.G., Golzarian, M.R., Mamarabadi, M., 2018. Detection and classification of citrus green mold caused by Penicillium digitatum using multispectral imaging. J. Sci. Food Agric. 98, 3542–3550. https://doi.org/10.1002/jsfa.8865.
Lei, Z., Huhman, D., Sumner, L.W., 2011. Mass spectrometry strategies in metabolomics. J. Biol. Chem. jbc-R111 https://doi.org/10.1074/jbc.R111.238691.
Lopéz-Pérez, M., Ballester, A., González-Candelas, L., 2015. Identification and functional analysis of *Penicillium digitatum* genes putatively involved in virulence towards citrus fruit. Mol. Plant Pathol. 16, 262–275. https://doi.org/10.1111/mpp.12179.
Macarisin, D., Cohen, L., Eick, A., Rafael, G., Belausov, E., Wisniewski, M., Droby, S., 2007. Penicillium digitatum suppresses production of hydrogen peroxide in host tissue during infection of citrus fruit. Phytopathology 97, 1491–1500. https://doi.org/10.1094/PHYTO-97-11-1491.
Marcet-Houben, M., Ballester, A.R., La Fuente, B., Harries, E., Marcos, J.F., González-Candelas, L., Gabaldón, T., 2012. Genome sequence of the necrotrophic fungus *Penicillium digitatum*, the main postharvest pathogen of citrus. BMC Genomics 13, 646. https://doi.org/10.1186/1471-2164-13-646.
Nguyen, D.D., Wu, C.H., Moree, W.J., Lamsa, A., Medema, M.H., Zhao, X., Gavilan, R.G., Aparicio, M., Atencio, L., Jackson, C., Ballesteros, J., Sanchez, J., Watrous, J.D., Phelan, V.V., van de Wien, C., Kersten, R.D., Mehnaz, S., De Mot, R., Shank, E.A., Charusanti, P., Nagarajan, H., Duggan, B.M., Moore, B.S., Bandeira, N., Palsson, B.O., Pogliano, K., Gutiérrez, M., Dorrestein, P.C., 2013. MS/MS networking guided analysis of molecule and gene clusters families. Proc. Natl. Acad. Sci. U. S. A. 110, E2611–E2620. https://doi.org/10.1073/pnas.1303471110.
Numata, A., Takahashi, C., Matsushita, T., Miyamoto, M., Kawai, K., Usami, Y., Matsumura, E., Inoue, M., Ohishi, H., Shingu, T., 1992. Fumiquinazolines, novel metabolites of a fungus isolates from a saltfish. Tetrahedron Lett. 33 (12), 1621–1624. https://doi.org/10.1016/S0040-4039(00)91690-3.
Panebianco, S., Vitale, A., Platania, C., Restuccia, C., Polizzi, G., Cirvilleri, G., 2014. Postharvest efficacy of resistance inducers for the control of green mold on important Sicilian citrus varieties. J. Plant Dis. Prot. 121, 177–183. https://doi.org/10.1007/BF03356507.
Perez, M.F., Ibarreche, J.P., Isas, A.S., Sepulveda, M., Ramallo, J., Dib, J.R., 2017. Antagonistic yeasts for the biological control of Penicillium digitatum on lemons stored under export conditions. Biol. Control 115, 135–140. https://doi.org/10.1016/j.biocontrol.2017.10.006.
Prusky, D., McEvoy, J.L., Saftner, R., Conway, W.S., Jones, R., 2004. Relationship between host acidification and virulence of *Penicillium* spp. on apple and citrus fruit. Phytopathology 94, 44–51. http://doi.org/10.1094/PHYTO.2004.94.1.44.
Scharf, D.H., Heinekamp, T., Brakhage, A.A., 2014. Human and plant fungal pathogens: the role of secondary metabolites. PLoS Pathog. 10 (1), e1003859. https://doi.org/10.1371/journal.ppat.1003859.
Sica, V.P., Raja, H.A., El-Elimat, T., Oberlies, N.H., 2014. Mass spectrometry imaging of secondary metabolites directly on fungal cultures. RSC Adv. 4, 63221–63227. https://doi.org/10.10139/c4ra11564c.
Smith, C.A., O'Maille, G., Want, E.J., Qin, C., Trauger, S.A., Brandon, T.R., Siuzdak, G., 2005. METLIN: a metabolite mass spectral database. Ther. Drug Monit. 27, 747–751.
Sun, F., Chen, G., Bai, J., Pei, Y., 2012. Two new alkaloids from a marine-derived fungus Neosartorya sp. HN-M-3. J. Asian Nat. Prod. Res. 14 (12), 1109–1115.
Yamazaki, M., Fujimoto, H., Okuyama, E., 1978. Structure determination of six fungal metabolites, tryptoquivaline E, F, G, H, I and J from Aspergillus fumigatus. Chem. Pharm. Bull. 26, 111–117.
Yamazaki, M., Okuyama, E., Maebayashi, Y., 1979. Isolation of some new tryptoquivaline-related metabolites from Aspergillus fumigatus. Chem. Pharm. Bull. 27, 1611–1617.
Zhang, T., Sun, X., Xu, Q., Candelas, L.G., Li, H., 2013. The pH signaling transcription factor PacC is required for full virulence in *Penicillium digitatum*. Appl. Microbiol. Biotechnol. 97, 9087–9098. https://doi.org./10.1007/s00253-013-5129-x.
Zhu, C., Sheng, D., Wu, X., Wang, M., Hu, X., Li, H., Yu, D., 2017. Identification of secondary metabolite biosynthetic gene clusters associated with the infection of citrus fruit by Penicillium digitatum. Postharvest Biol. Technol. 134, 17–21. https://doi.org/10.1016/j.postharvbio.2017.07.011.
Zin, W.W.M., Buttachon, S., Buaruang, J., Gales, L., Pereira, J.A., Pinto, M.M.M., Silva, A.M.S., Kijjoa, A., 2015. A new meroditerpene and a new tryptoquivaline analog from the algicolous fungus Neosartorya takakii KUFC 7898. Mar. Drugs 13 (6), 3776–3790. https://doi.org/10.3390/md13063776.