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# Secondary metabolites synthesized by *Stemphylium lycopersici* and *Fulvia fulva*, necrotrophic and biotrophic fungi pathogen of tomato plants

Rocio Medina[a,*,1], Mario E.E. Franco[a,1,2], César G. Lucentini[a], Janina A. Rosso[b], Mario C.N. Saparrat[c,d,e], Laura C. Bartel[a], Pedro A. Balatti[a]

[a] Centro de Investigaciones de Fitopatologías, CIDEFI, Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CICBA), Departamento de Ciencias Biológicas, Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, 60 y 119, CC 31, 1900 La Plata, Argentina
[b] Instituto de Investigaciones Fisicoquímicas Teóricas y Aplicadas (INIFTA), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Departamento de Química, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Diag. 113 y 64, CC 16 suc. 4, 1900 La Plata, Argentina
[c] Cátedra de Microbiología Agrícola, Departamento de Ciencias Biológicas, Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, 60 y 119, 1900 La Plata, Argentina
[d] Instituto de Fisiología Vegetal (INFIVE), Consejo Nacional de Investigaciones Científicas y técnicas (CONICET), Universidad Nacional de La Plata CCT-La Plata- Diag. 113 y 61, CC 327, 1900 La Plata, Argentina
[e] Instituto de Botánica Carlos Spegazzini, Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata, 53 CC 477, 1900 La Plata, Argentina

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ABSTRACT

The aim of this work was to study the volatile organic compounds (VOCs) profile and diffusible secondary metabolites (SMs) of *Stemphylium lycopersici* and *Fulvia fulva*, two fungal pathogens of tomato. *S. lycopersici* synthesizes and releases quantitatively more VOCs than *F. fulva*, probably due to the different type of interaction that each fungus establishes with tomato; nevertheless, *F. fulva* synthesized a specific and more diverse spectrum of VOCs. *S. lycopersici* released VOCs that triggering cell death in tomato leaves. Also, *F. fulva* synthesized an ample array of SMs but their biological roles remain to be elucidated.

## 1. Introduction

Tomato is one of the most consumed vegetable in the world [1], according to the Food and Agriculture Organization (FAO), approximately 60 million hectares produce 170.8 million tons [2]. In Argentina, the main areas cultivated with tomatoes are located in the provinces of Corrientes and Buenos Aires. In the latter one, production is carried out mostly in greenhouses [3], where relative humidity as well as temperature are high, favouring this the development of diseases provoked mostly by fungi [4].

Dothideomycetes is a class of fungi of the phylum Ascomycota that includes more than 25 orders of organisms adapted to a wide range of environments [5]. Among them, representatives of the genus *Stemphylium*, of the order Pleosporales, can establish pathogenic, saprotrophic, or endophytic relationship with a wide range of plant-host species [4,6]. *Fulvia fulva* (syn. *Cladosporium fulvum*) is also a dematiaceous fungus, but in the order Capnodiales, that has been considered a model for the study of plant-pathogen interactions since it behaves according to the gene for gene hypothesis [7,8].

Based on their life cycle, fungal plant pathogens can be classified as biotrophic, hemibiotrophic or necrotrophic [9,10]. *Stemphylium lycopersici* is the causal agent of grey leaf spot, which is a serious disease of tomato in Argentina [6]. *F. fulva* is the causal agent of leaf mould, a disease that affects mostly greenhouse grown tomatoes [11]. Both are foliar diseases that provoke reductions in plants leaf areas and, therefore, in yield. While *S. lycopersici* is a necrotrophic pathogen [12], *F. fulva* is a non-obligate biotrophic one [13].

*S. lycopersici* might provoke plant cell death through the synthesis and release of phytotoxic secondary metabolites [14,10]. Interestingly, the chemical structure, as well as the biological effects of these molecules have been elucidated only for few of them [15–17]. The other fungus, *F. fulva* secretes into the apoplast a set of effectors [18], such as

*Abbreviations:* AVR, avirulence; ECP, extracellular proteins; FEEMs, fluorescence–excitation-emission matrices; FAO, Food and Agriculture Organization; KI, Kovalts Index; PDA, potato dextrose agar; PDB, potato dextrose broth; ROS, resistance oxidative stress; SMs, secondary metabolites; SAR, systemic acquired resistance; SIR, systemic induced resistance; VOCs, volatile organic compounds

* Corresponding author.
*E-mail address:* rociomedinalp@gmail.com (R. Medina).
[1] Both authors contributed equally to this work.
[2] Current Address: Department of Biosystems Engineering, The University of Arizona, Tucson, AZ 85721, USA.

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avirulence AVR2 [19], AVR4 [20], AVR4E, AVR9 [21], and AVR5 [22], and extracellular ECP1, ECP2, ECP4 and ECP5 proteins [23]. Recently, new secondary metabolites synthesized by *F. fulva like* cladofulvin [24] and 1,8-dihydroxynapthalene-melanin [25,26] have been described.

Fungal secondary metabolites (SMs) are a wide range of low molecular weight organic compounds that are dispensable when microorganisms are cultivated *in vitro* but they provide adaptive advantages in nature. SMs might play relevant biological activities, that might affect food safety since some of them might be aflatoxins and/or trichothecenes or they might be useful of the pharmaceutical industry, since some SMs are key factors of pathogenicity [18]. It would be interesting to know whether their synthesis and/or secretion results in a compatible or incompatible interaction. Genes that code for the synthesis of SMs are arranged in clusters [27] and based on their biosynthetic pathway they might be classified as polyketides, non-ribosomal peptides, hybrid polyketide synthase/non-ribosomal peptide synthetase, terpenes or alkaloids [28]. According to their physicochemical nature, they can be volatile, soluble or insoluble compounds. Volatile ones might play an important role in long-distance biological interactions [29]. Several researchers found that many plants respond to a specific set of pathogen-derived volatile organic compounds (VOCs), which trigger direct and/or indirect defence responses [30,31]. Moreover, differential responses have been found regarding the nature of the plant stress, for example when the plant is attacked by a necrotrophic fungal pathogen, systemic induced resistance (SIR) might be triggered, while the systemic acquired resistance (SAR) is triggered by an environmental stress or a biotrophic fungi [32–35]. The information regarding the synthesis and/or secretion of VOCs by *S. lycopersici* and *F. fulva* and their role in plant pathogen interactions is lacking, therefore, the aim of this work was to analyse the profile of VOCs synthesized and released by a necrotrophic and a non-obligate biotrophic fungus that provoke diseases on tomato leaves as well as the soluble SMs that alter the plant physiology.

## 2. Materials and methods

### 2.1. Fungal material

The isolates of *Stemphylium lycopersici* (CIDEFI 213 and CIDEFI 216) and *Fulvia fulva* race-2 (CIDEFI 300) used in this work belong to the culture collection of the Centro de Investigaciones de Fitopatología (CIDEFI), Universidad Nacional de La Plata (UNLP) that have already been characterized [3,26,6]. The strains of *S. lycopersici* differ in their virulence.

### 2.2. Growth conditions

Fungal cultures of *S. lycopersici* CIDEFI 213 and 216 [6] were grown on potato dextrose agar (PDA) medium at 24 °C in the darkness for 7 days and *F. fulva* CIDEFI 300 under the same conditions for 14 days respectively [3]. Two agar plugs (diameter, 5 mm) from actively growing cultures were picked with a sterile glass borer and placed in a glass headspace vial (10 mL) filled with 3.5 mL of potato dextrose broth (PDB) medium (Sigma-Aldrich, Australia), which was sealed with a silicone septum cap. Vials were incubated in a rotary shaker at 150 rev. $min^{-1}$ at 24 °C in the dark for 7 in the case of *S. lycopersici* and 14 days in the case of *F. fulva*.

### 2.3. Volatile organic compounds (VOCs): extraction, identification and quantification

The VOCs profiles of fungi were analysed by gas chromatography–mass spectrometry (GC–MS) using a HP CGC 6890/MS Agilent 5975C VL gas chromatograph - mass spectrometer equipped with a ZB-5HT Inferno fused silica capillary column (30 m, 0.25 mm i.d., 0.25 µm, Phenomenex, Inc). A solid-phase microextraction fiber coated with 65 µm polydimethylsiloxane/divinylbenzene was used to extract VOCs from the stand still vials, that were incubated for an hour at 30 °C. After injection, the compounds were desorbed for 5 min in a splitless injector at 250 °C. The oven temperature was held at 40 °C for 2 min, then raised to 200 °C at 10 °C $min^{-1}$ and 250 °C at 15 $min^{-1}$, then the temperature was hold for 5 min. Helium was the carrier gas flowing at 1 mL $min^{-1}$. Compounds were identified by matching their mass spectra using the NIST Mass Spectra Search Program with NIST05 and Adams (Identification of Essential Oil components by Gas chromatography/Mass spectrometry, 4th Edition) libraries and using Kovalts index (KI) in reference to n-alkanes. The background of PDB media un-inoculated was analysed as control.

### 2.4. Spectrofluorometric supernatants of fungal cultures

An aliquot of the supernatants of fungal cultures grown on PDB were filtered through a 0.45 µm (pore) membrane to perform the spectrofluorometric analysis. The absorption spectra were measured on a Shimadzu UV-1800 at room temperature in quartz cells with 1.0 cm optical path length between 200 and 800 nm. The fluorescence–excitation-emission matrices (FEEMs) were determined using a Single-Photon Counting equipment FL3 TCSPC-SP (Horiba Jobin Yvon). FEEMs were generated by collecting the data of successive emission spectra according to described by Medina et al. [36].

### 2.5. Virulence assay

An aliquot of supernatants of fungal cultures grown on PDB were sterilized by passing the culture supernatant through a sterile 0.22 µm membrane. Virulence assayes on detached leaves were carried out using unfiltered and filtered fungal culture supernatants as described [6]. As negative control leaflets were treated with an aliquot of sterilized water and un-inoculated PDB. The positive control was an unfiltered supernatant of fungal cultures grown on PDB for 7 and 14 days according the fungal specie (*See* section 2.2). Petri dishes were sealed with Parafilm and were incubated for a week at 25 °C. The average of the lesion was determined after 7 days post inoculation and was measured by means of the image analysis software Assess 2.0 [37]. The experiment was carried out with nine replicates of one leaflet per replicate inoculated with each strain. Data were analysed by analysis of variance (ANOVA) after the Tuckey test ($p < 0.05$), with InfoStat version 2015 l [38].

## 3. Results and discussion

### 3.1. VOCs released by Stemphylium lycopersici and Fulvia fulva

Tomato is affected both by necrotrophic as well as biotrophic fungal pathogens that synthesize soluble and volatile secondary metabolites that alter plant physiology. The VOCs synthesized by *S. lycopersici* CIDEFI 213, CIDEFI 216 and *F. fulva* CIDEFI 300 are shown in Additional Figs. 1–3. The integrated areas were 1.13 $exp^8$ and 6.18 $exp^7$ for *S. lycopersici* strains CIDEFI 213 and CIDEFI 216, respectively and 1.72 $exp^7$ for *F. fulva* CIDEFI 300. These results showed that *S. lycopersici* produces a larger quantity of VOCs than *F. fulva*, which might be associated with their necrotrophic and non-obligate biotrophic nature, respectively [18,6].

Both isolates of *S. lycopersici* and the only one strain CIDEFI 300 of *F. fulva* produced a total of 25 volatile compounds (Table 1 and Table 2). It has already been demonstrated that *Stemphylium* species synthesize a wide array of SMs that might play, either alone or in mass, a key role during host plant infection [39] and also might explain their necrotrophic behaviour. Interestingly, among cultures of *F. fulva* the only SMs so far identified were cladofulvin [40] and 1,8-dihydroxynaphtalene-melanin [25,26]. However, our chromatographic analysis showed that *F. fulva* CIDEFI 300 synthesized 18 other SMs (Table 1). In line with this finding, Collemare et al. [18] reported that the genome of

**Table 1**
Relative abundance [%] of volatile organic compounds (VOCs) identified by means of a GC–MS analysis in cultures of *S. lycopersici* CIDEFI 213 and CIDEFI 216 strains grown in PDB media. Compounds presented in bold letters also were detected in cultures of *F. fulva* strain CIDEFI 300.

| RT (min) | KI | Compound | CIDEFI 213 | CIDEFI 216 |
|---|---|---|---|---|
| 1.64 | 624.27 | Ethyl alcohol | 14.12 | 21.08 |
| 1.74 | 629.54 | **Acetone** | 0.34 | – |
| 2.45 | 669.56 | 2-Methyl-1-propanol | 4.94 | 6.81 |
| 3.72 | 740.64 | **Isoamyl alcohol** | 40.08 | 14.64 |
| 3.78 | 743.89 | 2-Methyl-1-butanol | 25.45 | 41.46 |
| 5.80 | 857.12 | **Furfuryl alcohol** | 0.91 | 1.09 |
| 8.13 | 989.59 | 6-Methyl-5-hepten-2-one | 0.14 | – |
| 8.72 | 642.25 | No identified Nist05 | 0.18 | – |
| 8.81 | 701.95 | 2-Ethyl-1-hexanol | 0.20 | – |
| 8.92 | 711.54 | Benzyl alcohol | 0.46 | 0.61 |
| 10.21 | 696.60 | **Phenethyl alcohol** | 13.19 | 14.31 |

RT: retention time. KI: Kovalts index.

**Table 2**
Relative abundance [%] of volatile organic compounds (VOCs) identified by means of a GC–MS analysis in cultures of *F. fulva* CIDEFI 300 strain grown in PDB media. Compounds presented in bold letters also were detected in cultures of *S. lycopercisi* strains CIDEFI 213 and CIDEFI 216.

| RT (min) | KI | Compound | CIDEFI 300 |
|---|---|---|---|
| 1.74 | 629.54 | **Acetone** | **3.86** |
| 3.59 | 733.18 | Methyl trimethylacetate | 8.52 |
| 3.73 | 741.09 | 3-Methyl-3-buten-1-ol | 0.62 |
| 3.72 | 740.64 | **Isoamyl alcohol** | 20.13 |
| 4.25 | 770.12 | Toluene | 0.65 |
| 4.64 | 792.43 | 1-octene | 2.04 |
| 5.56 | 843.83 | 3-Hexanone, 4-methyl- | 1.28 |
| 5.80 | 857.12 | **Furfuryl alcohol** | 1.37 |
| 5.90 | 862.78 | Ethylbenzene | 0.73 |
| 6.05 | 871.19 | p-Xylene | 0.78 |
| 6.13 | 875.73 | 4-Heptanone | 0.87 |
| 6.31 | 885.65 | Bromoform | 0.52 |
| 6.43 | 892.60 | Styrene | 6.77 |
| 8.10 | 988.05 | 3-Octanone | 5.36 |
| 9.05 | 720.15 | Hexanoic acid, 2-ethyl-, methyl ester | 1.03 |
| 9.83 | 719.66 | 2-Nonanone | 9.34 |
| 10.21 | 696.60 | **Phenethyl alcohol** | 1.58 |
| 12.67 | 694.13 | No identified Nist05 | 34.54 |

RT: retention time. KI: Kovalts index.

*F. fulva* strain race 0WU harbours 23 gene clusters coding for putative SMs and highlighted that it might be a way that too a large number considering the biotrophic nature of the fungus. These results argue against the hypothesis that biotrophic fungi evolved and developed such a strategy of interaction with plants by losing gene coding for SMs.

We identified organic compounds that are involved in virulence, *quorum-sensing*, differentiation and other processes within fungi that might alter plant-fungal interactions. Isoamyl, phenethyl and furfuryl alcohols as well as acetone were synthesized by cultures of both fungal species (Fig. 1). Isoamyl alcohol was the main common organic compound among the strains, with a relative abundance > 14.6%. Alcohol production has been studied mostly in *Saccharomyces cerevisiae* that synthesizes a wide array of alcohols, among them fusel alcohols that result from the amino acids fermentation [41]. Hazelwood et al. [42] found that when fungi are cultured under nitrogen limiting conditions, frequently they synthesize aromatic compounds such as isoamyl and isobutyl alcohols, that have been associated with the activation of processes related with virulence [33]. Amino acids like valine, leucine, isoleucine, methionine, and phenylalanine are assimilated *via* the Ehrlich pathway. Interestingly, compounds synthesized through this pathway might trigger *quorum-sensing*, which leads to the induction of differentiation and probably yeast cells adaptation to the environment [41,42]. Leucine, under nitrogen limiting conditions, is the precursor of

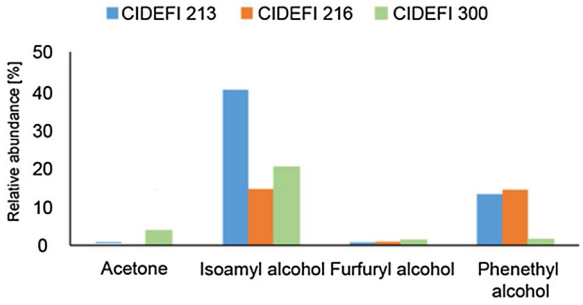


**Fig. 1.** Relative abundance [%] of volatile organic compounds (VOCs) identified by means of a GC–MS analysis in cultures of *S. lycopersici* CIDEFI 213 and CIDEFI 216 strains and in cultures of *F. fulva* CIDEFI 300 strain grown in PDB media that were found within the three studied strains.

isoamyl alcohol [43], a compound that provokes cell elongation, pseudohyphal growth and chitin synthesis [42]. In line with this, also germination of fungal spores is stimulated by isoamyl alcohol [44]. Phenethyl alcohol is also synthesized and released by *S. lycopersici* (> 13.19%) and *F. fulva* (1.58%) studied isolates and it might work as a growth controlling compound like in *Candida albicans* [45]. Furfuryl alcohol, another compound synthesized and released by both fungal species, at lower concentrations than 1% is a reduced less reactive derivative of furfural [46]. Under microaerophilic conditions furfural triggers reactive oxygen species (ROS) production, unlike furfuryl alcohol, that provokes a reduction of ROS and therefore cells are not damaged [47], thus suggesting that this type of compounds might play a role in symptom development on tomato leaves.

Acetone was another compound detected in cultures of both fungal pathogens but it was produced by only one *S. lycopersici* strain, particularly in CIDEFI 213 culture in a low proportion (0.34%) compared to cultures of *F. fulva* (3.86%). Several authors described the production of acetone–butanol–ethyl alcohol through a fermentation process led by solventogenic *Clostridium* species [48–50].

In summary, when *S. lycopersici* and *F. fulva* are grown under oxygen and/or nitrogen limiting conditions, isoamyl alcohol, phenethyl alcohol, furfuryl alcohol and acetone synthesis is triggered. These VOCs might be turning on transduction signal pathways that incidentally led to mycelial growth autostimulation and/or inhibition of another putative competitor in a particular environment. All these considerations suggest that oxygen and nitrogen might play a key regulatory role in *S. lycopersici* and *F. fulva* volatile compound synthesis.

Both phytopathogens, *S. lycopersici* and *F. fulva,* synthesize SMs like some of them are VOCs that include primarily alcohols, ketones and aldehydes [33] that though they are not essential for fungal growth, they may provide adaptive advantages in nature [51,52]. In addition to this, *S. lycopersici* also has the potential to secrete a broad host range of cell-wall-degrading enzymes and toxins, which is probably related to its necrotrophic capacity [53]. On the contrary, *F. fulva*, a non-obligate biotrophic fungus [11], is thought to be under an evolutionary process of a convergent loss of genes coding for secondary metabolic enzymes, which might occur through the reduction of genes encoding specific toxin transporters. This might additionally reduce the ability of *F. fulva* to secrete enzymes and toxins compared to necrotrophic fungi, which is in line with its ecological behaviour when it interacts with tomato [33].

### *3.2. VOCs synthesized by Stemphylium lycopersici CIDEFI 213 and CIDEFI 216 strains*

The relative abundance of VOCs released by *S. lycopersici* CIDEFI213 and 216 cultures is presented in Fig. 2. Both isolates synthesized and released 6 compounds Ethyl alcohol, 2-methyl-1- propanol, 2-methyl-1-butanol, 6-methyl-5-hepten-2-one, 2-ethyl-1-hexanol and benzyl alcohol that were not detected in *F. fulva* CIDEFI 300 cultures. These

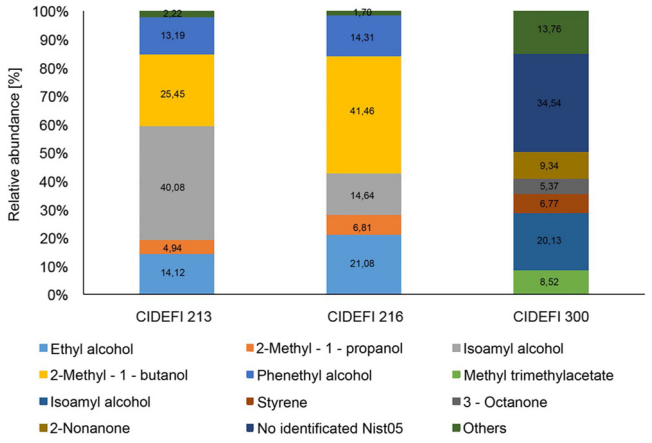


**Fig. 2.** Relative abundance [%] volatile organic compounds (VOCs) identified by means of a GC–MS analysis in cultures of *S. lycopersici* CIDEFI 213 and CIDEFI 216 strains and in cultures of *F. fulva* CIDEFI 300 strain grown in PDB media. Compounds with relative abundance < 5% were grouped as "Others".

compounds, together or alone, might be involved in several biological process.

The first one, ethyl alcohol, is the main compound released by several fungi [41]. 2-methyl-1- propanol, has fungivore attractant activity [54]. Recently, Li et al. [55] reported that strains of *Verticillium* also produce 2-methyl-1-propanol and phenylethyl alcohol, though their biological role is unknown [56,57]. In a few dimorphic fungi, including plant pathogens, *quorum-sensing* mechanisms have been identified that might be triggering pathogenic processes. Berrocal et al. [58] suggested that 2- methyl-1-butanol might be mediating *quorum-sensing* dependent mechanisms in fungi of the genus *Ophiostoma*. This compound also has been found in cultures of *Aspergillus niger*, *A. versicolor* and *Penicillium brevicompactum* [59]. Furthermore, Mercier and Jimenez [60] found that *Muscodor albus* synthesizes and releases a mixture of 2- methyl-1-butanol and isobutyric acid, which appears to successfully control postharvest plant diseases. In addition to this, Hung et al. found that 2- methyl-1-butanol, stimulated chlorophyll synthesis in *A. thaliana* plants [57]. In summary, all these findings suggest that 2-methyl-butranol might play a key role in *quorum-sensing* and chlorophyll synthesis in plants that might be critical in the *Stemphylium*-Tomato interaction. The fact that 2- methyl-1-butanol was not detected in cultures of *F. fulva* raises a question regarding either the role of such compounds in *quorum-sensing* during *F. fulva*-Tomato interaction or on *quorum-sensing* itself.

On the other hand, two compounds, 6-methyl-5-hepten-2-one and 2-ethyl-1-hexanol only were released by cultures of *S. lycopersici* CIDEFI 213. The former one induced germination of urediniospores of *Puccinia graminis*, *P. coronata*, *P. sorghi* and *P. recondite* [44] and also has been associated to citral detoxification in *Penicillium expansum* [61,62]. The other one, 2-ethyl-1-hexanol, triggered inhibition of mycelial growth and spore germination [63,64] and also was synthesized and released by isolates of *Pseudomonas* sp., inhibiting *Sclerotinia sclerotiorum* spore germination and growth [65]. Hence, the role of 2 ethyl-1-hexanol deserves further attention regarding its biological role in plant-microbe interactions.

Another VOC found in cultures of *S. lycopersici* was benzyl alcohol, a compound that has antifungal activity [63,66] though the most important difference between *S. lycopersici,* CIDEFI 213 and CIDEFI 216 isolates, were their ability to synthesize isoamyl alcohol and 2-methyl-1-butanol (*sec*- butyl carbinol), particularly the latter one was abundant in cultures of CIDEFI 216, which might be associated with its virulence [6].

These results confirmed that *S. lycopersici* synthesizes a set of SMs with a wide array of biological roles like antagonisms, which might confer *S. lycopersici* a competitive advantage over other organisms. Strikingly, this does not seem to be the case of *F. fulva,* a fungal pathogen that interacts with tomato in a different way that *S. lycopersici*.

### 3.3. VOCs synthesized by Fulvia fulva CIDEFI 300 strain

*Fulvia fulva* also synthesize and release VOCs (Fig. 2). The compound represented the most (34.5%) has a retention time of 1267 min, KI 694.13 and a mass of approximately 198.2 atomic mass unit (Fig. 3), however, we were unable to find a compound with similar characteristics in the NIST05 and Adams libraries. Also, *F. fulva* produced 2-nonanone (9.4%), a compound with antifungal activity [44,63,67], that also is synthesized by bacteria [68,69]. Another VOCs synthesized and released by *F. fulva* were methyl trimethylacetate (8.5%, methyl 2,2-dimethylpropionate) and styrene (6.8%). The former compound might be the precursor of other metabolites such as dimethylpropionate, a compound that protects plants from pathogens by inducing systemic resistance [70]. On the other hand, Furia and Bellanca, Li et al. and Wen et al. found that styrene is indicative of the presence of pathogens in fruits [71–73]. The styrene secreted by *Penicillium expansum* significantly reduced the attraction exerted by pieces of pine twigs upon pine weevil's (*Hylobius abietis*) cut [74]. *F. fulva* also synthesized and released 3- octanone (5.4%), a compound mostly related with mouldy, earthy, mushroom flavours [75]. In addition to this, 3-octanone at low concentration repelled *Megaselia halterata* [76] and at high ones, inhibited fungal growth [77]. The 3-octanone of *Cladosporium* spp. reduced disease symptoms in *A. thaliana* infected with *P. syringae pv. tomato* [78] mostly because it induced the systemic resistance of plants to diseases. Furthermore, 3-octanone proved to be phytotoxic on *A. thaliana*, where it triggered an oxidative burst [79,43]. Also, other minor compounds were detected within cultures of *F. fulva,* like 1-

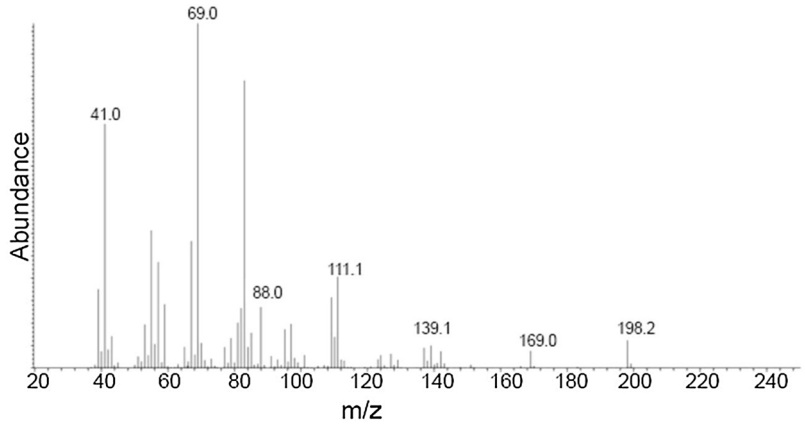


**Fig. 3.** Mass spectra of the more abundant compound detected by headspace GC–MS from cultures of *F. fulva* CIDEFI 300 strain grown on PDB media for 14 days.

octene (2.0%), 3- hexanone, 4 methyl- 3- hexanone (1.3%), hexanoic acid, 2- ethyl-, methyl ester (1.0%), 4- heptanone (0.9%), p- xylene (0.8%), ethylbenzene (0.7%), toluene (0.6%), 3- methyl-3-buten-1-ol (0.6%) and bromoform (0.5%). Although *Cladosporium cladosporioides* produced relatively high concentrations of 1-octene its biological role is unknown [80]. Three- hexanone enhanced growth of *A. thaliana* [69], while 4-methyl- 3- hexanone was found to repel insects [81]. It is well known that plant defensive mechanisms, like SIR and/or SAR contribute to plant health since they conform the plant immune system [82]. Fungal VOCs, like caryophyllene, m-cresol, methyl benzoate, 3-octanone, 1-octen-3-ol, 6-pentyl-a-pyrone activated induced systemic resistance in plants [83]. *F. fulva* synthesize 3-octanone that based on the findings of Martínez-Medina et al. [84] might play additional roles in plants like activating any of the mechanisms that form the plant immune system.

Our results demonstrated that *F. fulva* synthesizes and secretes antimicrobial compounds against putative competitive microorganisms as well as insects, contributing in this way to its own growth, which possess and environmental advantage for the organism. In accordance with its biotrophic behaviour it seems that the pathogen, secrete compounds that not only stimulate plant growth and health but they also act upon competitors as well as insects.

### 3.4. Study of biological and chemical characteristic of supernatants of fungal cultures

The solubility and vapour pressure at 20 °C of VOCs detected are listed on Additional Table 1. Compounds with high water solubility can be detected by photochemical techniques, whether they and had a biological implication on tomato plants was evidenced by an *in vitro* assay using leaflets.

Regarding the detached leaf assay, both filtered and unfiltered supernatants of *S. lycopersici* provoked the development of a necrotic area quite similar in appearance to grey leaf spot symptoms (Fig. 4 and Additional Fig. 4). Franco et al. [6] described that CIDEFI 216 was more virulent than CIDEFI 213, when the detached leaf assay was carried out using conidia. UV–vis analysis showed that the supernatant of CIDEFI 213 strain has an absorption region between λ 240–320 nm with a maximum on λ 275 nm. The supernatant of CIDEFI 216 had the same absorption region that CIDEFI 213 but larger amplitude (Fig. 5.A). Apart from these differences on the UV–vis spectra, CIDEFI 213 and CIDEFI 216 strains did not showed differences in fluorescence compared to the control (data not showed). It is possible that the intrinsic fluorescence of the PDB prevent us from seeing the differential fluorescence provoked by compounds secreted by the strains. Furthermore, apparently both *S. lycopersici* strains synthesized similar soluble compounds. Even though CIDEFI 216 secreted more compounds, at least

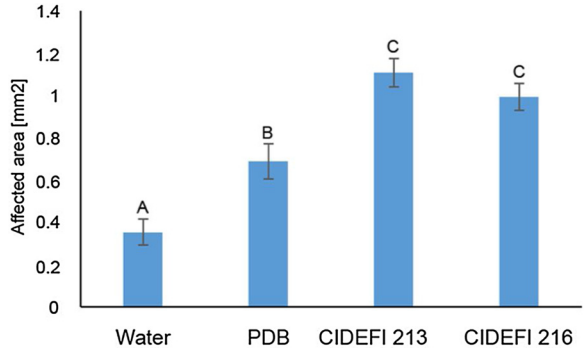


**Fig. 4.** Average size of the affected area development on detached leaf by filtered supernatant from cultures of *S. lycopersici* grown on PDB media for 7 days. Values are the average of nine replicates. For the same parameter, the mean values followed by different letters are significantly different ($p < 0.05$). Error bars indicates standard deviation.

based on the amplitude of the absorption spectra, most probably not all of them provoked necrosis. In addition to this, both isolates secreted compounds that provoke the death of plant tissue. Hence, the major virulence of isolate CIDEFI 216 described by Franco et al. [6] was most probably due, not only to the presence of low weight metabolites (including VOCs), but also to their interaction with enhancers like VOCs (probably with lower solubility and/or higher vapour pressure) or protein effectors that were synthesized during plant-pathogen interactions.

Last but not least, unfiltered supernatants of *F. fulva* cultures developed typical symptoms of tomato leaf mould on leaves after 7 days of inoculation, which was not observed with filtered supernatants, a response that might be related with the non-obligate biotrophic interaction established between *F. fulva* and Tomato. In addition, UV–vis analysis showed that supernatant of CIDEFI 300 strain has a differential absorption spectra compared to PDB between λ 240–280 nm (Fig. 5.B). This change was accompanied with changes in fluorescence emission (Fig. 6). The peak with maximum of fluorescence on $\lambda_{exc}$ 325- $\lambda_{em}$ 420 was more intense in CIDEFI 300 strain. Based on our results, we can infer that the photochemical characteristic of CIDEFI 300 supernatant is a wide array of secreted compounds. In addition, the absence of leaf mould symptoms on detached leaves suggest that these compounds, including identified and unidentified VOCs, do not have the ability to produce disease, at least at the concentration assayed.

## 4. Conclusions

In summary, *S. lycopersici* synthesizes and releases quantitatively more VOCs than *F. fulva*, probably due to the different type of interaction that each fungus establish with Tomato; nevertheless, *F. fulva* synthesized a specific and more diverse spectrum of VOCs.

Production of furfuryl alcohols and derivative compounds by *S. lycopersici* and *F. fulva* may be associated with symptoms development on tomato leaves. In addition, *F. fulva* produces compounds that might contribute to tissue colonization. Since the biological role of the main VOCs released by *F. fulva,* including an unidentified dominant one, remains unknown, future studies should be aimed at elucidating their roles.

This is the first report of the VOCs profiles produced by *S. lycopersici* and *F. fulva.* Hence, this works provides additional information about their ability to synthesize SMs and, thus, (it may shed some light on) the interaction with their hosts.

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## Declaration of Competing Interest

The authors declare that they have no competing interests.

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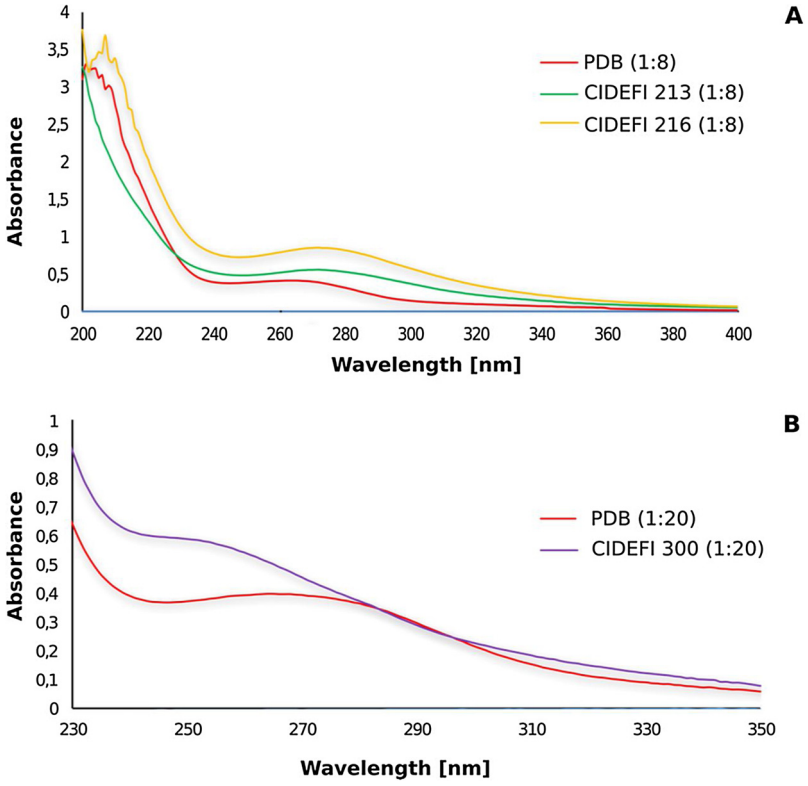


**Fig. 5.** UV–vis analysis of filtered supernatants of cultures. **A.** From *S. lycopersici* CIDEFI 213 and CIDEFI 216 strains. **B.** From *F. fulva* CIDEFI 300 strain.

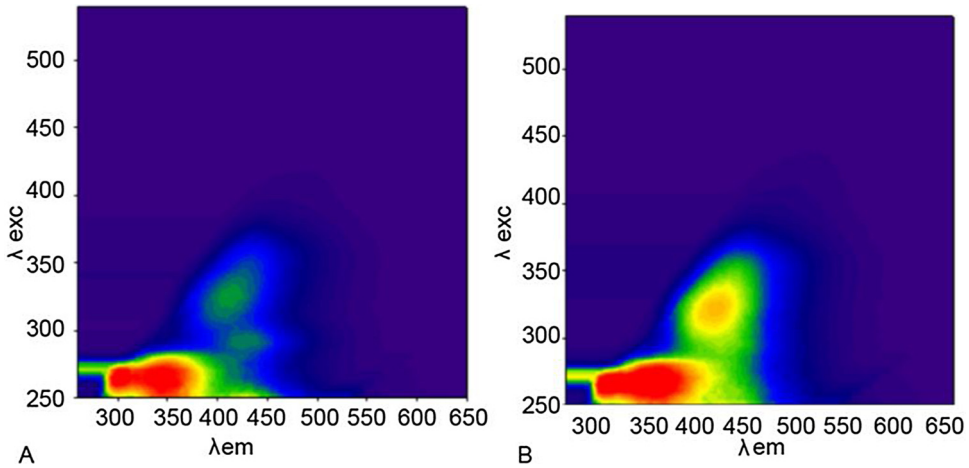


**Fig. 6.** Fluorescence–excitation-emission matrix (FEEMs). **A.** Un-inoculated PDB media (1:20). **B.** *F. fulva* CIDEFI 300 strain (1:20). Graphics were made using the same colour intensity scale.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cpb.2019.100122.

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