Identification of the Main Toxins Isolated from *Fusarium oxysporum* f. sp. *pisi* Race 2 and Their Relation with Isolates Pathogenicity

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ABSTRACT

Fusarium oxysporum f. sp. *pisi* (*Fop*) is a pathogen of field pea inducing severe vascular wilt worldwide. Plant resistance to race 1, 5 and 6, producing wilt symptoms, is conferred by a single dominant gene, while resistance to race 2, which gives near-wilt symptoms, have been recently showed to be quantitative. Among the virulence factors reported to play a role in the infection process, toxin production is one of the best studied. Thus, five race 2 isolates have been investigated for toxins production in vitro and their relation to isolates pathogenicity. All the isolates produced different amounts of fusaric and 9,10-dehydrofusaric acids. The content of the two toxins has been quantitated and correlated with the pathogenicity and aggressiveness of isolates on field pea. Results suggested that toxin production is an important determinant of *Fop* race 2 pathogenicity.

KEYWORDS: *Fusarium oxysporum* f. sp. *pisi*; *Pisum sativum*; near wilt; phytotoxins; fusaric acids

INTRODUCTION

Fusarium oxysporum f.sp. *pisi* W.C. Snyder & H.N. Hansen. (*Fop*) is an important pathogen causing vascular wilt of field pea (*Pisum sativum* L.) worldwide.[1] Four different races of *Fop,* races 1, 2, 5 and 6 have been described.[2] Races 1 and 2 have been reported in every country where peas are grown, while races 5 and 6 are, to date, only important in western Washington State.[2] Plants infected with race 2 are most often scattered throughout the field rather than being concentrated in specific areas as observed with the other races, which is described as *near wilt*.[1] Resistance to *Fop* race 1, 5 and 6 is conferred by single dominant gene while resistance to race 2 have been recently shown to be quantitative.[2, 3]

Several virulence factors have been reported to play a role at different stages of the infection process to induce disease and counteract the plant defence reaction in several formae speciales (ff. spp). of *Fusarium oxysporum*.[4] However, these studies have not targeted the virulence factors of *Fop.* One of the best studied virulence factors is the fusaric acid, **1** (Figure 1). Fusaric acid is a non-specific toxin produced by many *Fusarium* species.[5, 6] At high concentration it induces many physiological responses in plant cells including alteration of cell growth, mitochondrial activity and membrane permeability while at lower concentration it can trigger plant defence reactions and programmed cell death.[7, 8] Fusaric acid was also shown to induce wilt symptoms on pepper and cucumber.[9, 10] Thus **1** is considered to participate in *F. oxysporum* pathogenicity by reducing plant cell viability. However, **1** was also detected within plant tissue colonized by non-pathogenic isolates which questions the exact importance of **1** during the infection process.[7] Thus, the role of **1** in *F. oxysporum* pathogenicity is still under debate. Apart from fusaric acid, some *F. oxysporum* isolates can produce additional toxins such as beauvericin, enniantin B, bikaverin, moniliformin, fumonisin and trichothecenes[11-14] that can also contribute to their pathogenicity. Toxins produced by *Fop* and their potential function in *Fop* pathogenicity is still unknown. To improve understanding on *Fop* pathogenicity, the main toxins of several isolates of *Fop* race 2 were identified and quantitated.

MATERIALS AND METHODS

General Experimental Procedures. IR spectra were recorded as deposited glass film on a Perkin-Elmer (Norwalk, CT) Spectrum One FT-IR spectrometer and UV spectra were measured in MeCN on a Perkin-Elmer Lambda 23 UV/Vis spectrophotometer. ^{1}H NMR spectra were recorded at 600 or 400 MHz, in CD_3OD, on Bruker (Kalsruhe, Germany) spectrometers. The same solvent was used as internal standard. ESI and APCI MS spectra were recorded on an Agilent Technologies (Milan, Italy) 6120 Quadrupole LC/MS instrument. Analytical and HPLC grade solvents for chromatography were purchased from Carlo Erba (Milan, Italy). All other analytical grade chemicals were purchased from Merck (Darmstadt, Germany). Analytical and preparative thin layer chromatography (TLC) were performed on silica gel (Kieselgel 60, F_{254}, 0.25 and 0.5 mm respectively) (Merck, Darmstadt, Germany) or reverse phase (KC18 F_{254}, 0.20 mm) (Whatman, Maidstone, UK) plates. The spots were visualized by exposure to UV radiation (254 nm), or by spraying first with 10% H_2SO_4 in MeOH and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110 °C for 10 min, or by exposure to iodine vapours. The HPLC system (Shimadzu, Tokyo, Japan) consisted of a Series LC-10AdVP pump, FCV-10AlVP valves, SPD-10AVVP spectrophotometric detector and DGU-14A degasser. The HPLC column used was 250 x 4.6 mm i.d.; 5 μm high-density Nucleosil 100-5 RP18 (Macherey-Nagel, Duren, Germany) preceded by an in-line guard column (Alltech, Sedriano, Italy). Water was HPLC quality, purified in a Milli-Q system (Millipore, Bedford, MA, USA). Disposable syringe filters, Anotop 10-0,2 μm, were purchased from Whatman (Maidstone, UK). Fusaric acid was purchased from Sigma (St. Louis, MO, USA)

The methyl esters of **1** and **2** were prepared by diazotization of the corresponding acids as previously reported.[16]

Purification of 9,10-dehydrofusaric acid. 9,10-dehydrofusaric acid, **2** (Figure 1) was purified from fungal culture filtrates of *Fusarium nygamai* as previously described by Capasso et al., 1996.[15] Briefly, culture filtrates were acidified up to pH 2 and exhaustively extracted with

EtOAc. The organic extract was purified by combination of column and TLC on silica gel and reverse phase yielding **2** as a homogeneous amorphous solid (121,6 mg/L).

Fungal Strains, Culture Medium and Growth Conditions. The *Fusarium oxysporum* f. sp. *pisi* race 2 isolates F42 and F69 were kindly provided by Dr. W. Chen (USDA-ARS, Pullman, USA). The *F. oxysporum* f. sp. *pisi* strain CBS 127.73 NRRL36628 (*Fop*1) was provided by CBS-KNAW Fungal Biodiversity Center (Utrecht, The Netherlands). In addition, the strain Pt1 and Arg3 were isolated from wilted pea plants collected at Alvaiázere, Portugal and Setif, Algeria respectively. Isolation of fungal colonies on surface-sterilized wilted pea fragments was performed as described previously[3] and maintained as a single-spore colony. They have been deposited in the collection of Institute for Sustainable Agriculture, IAS-CSIC (Córdoba, Spain). The fungal strains were stored as microconidial suspensions at –80 ºC in 30% glycerol. For microconidia production, cultures were grown in potato dextrose broth (PDB) (Difco, Detroit, MI) at 28 ºC in a shake culture set at 170 rpm.[17] For toxins production, Erlenmeyer flasks (500 mL) containing 200 mL of Czapek-Dox medium (5% glucose, 0.1% yeast extract, 0.05% K_2HPO_4, 0.2% $NaNO_3$, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.001% $FeSO_4 \cdot 7H_2O$), were inoculated with 200 µL of fresh *Fop* microconidia at 10^9 conidia/mL and incubated under shaking at 28 °C for 7 days. The content of the flask was filtered on cheesecloth and centrifuged at 7,000 rpm for 10 min. The supernatant containing the secreted *Fop* toxins was then frozen at -80 ºC and lyophilized before further analysis. Morphological characterisation of each strain was performed at X40 and X63 magnification with a bright light microscope with Nomarsky filter.

DNA Extraction, PCR Amplifications and Sequencing. Genomic DNA was extracted from *F. oxysporum* mycelium following a previously reported protocol.[18] Molecular characterization of each *Fop* strain was performed by analysis of the internal transcribed spacers (ITS) and the 5′ intron-rich portion of the elongation factor alpha (EF-alpha). ITS and EF-alpha sequences were obtained by PCR amplification with primers ITS-1/ ITS-4[19] and EF-1/EF2[20]

106 respectively. Each 50 µL reaction mixture contained 50 ng of template DNA, 2 units of BioTaq
107 DNA polymerase (Bioline, London, UK), 1× PCR buffer, 2 mM $MgCl_2$, 200 µM dNTPs, and 0.3
108 µM of each primer. The PCR amplifications were performed on a MyCycler (Biorad, Hercules, CA)
109 thermocycler as follows: (i) for ITS, 94 °C for 2 min, 40 cycles at 94 °C for 1 min, 55 °C for 2 min,
110 and 72 °C for 2,5 min followed by a final step at 72 °C for 10 min; ii) for EF-alpha, 94 °C for 5
111 min, 30 cycles at 94 °C for 35 s, 52 °C for 35 s, and 72 °C for 1 min followed by a final step at 72
112 °C for 10 min. All amplifications were purified with the PCR cleanup kit of QIAgen and cloned in
113 pGEMT vector (Promega, Madison, WI). Two positive clones per amplicon were sequenced by
114 STABVida (Setubal, Portugal) using the pGEMT vector specific primers SP6 and T7.

115 **Sequence Data Analysis.** Reads of each sequence were assembled and manually corrected
116 with Chromaspro 1.7.5 (Technelysium Pty Ltd., South Brisbane, Australia). Sequences were used in
117 BLAST[21] searches against the GenBank[22] and Mycobank[23] databases to identify the most similar
118 sequences available in the databases. Pair-wise alignments were performed with the Neddleman-
119 Wunsh algorithm (Needle method) implemented at EMBL-EBI webserver[24]. All DNA sequences
120 have been deposited in GenBank (Table S1).

121

122 **Plant Materials.** Assignment of the different strains to a specific race of *F. oxysporum* f. sp.
123 *pisi* was performed by testing the pathogenicity profile of these isolates on a set of differential pea
124 lines (Table 1).[1,3] The comparison of the aggressiveness of each strain was performed on *P. sativum*
125 cv. 'Messire'. To determine the effect of fungal culture filtrates or their corresponding organic
126 extracts, seven pea accessions with a wide range of response to *Fop* race 2 were used including the
127 susceptible accessions JI1213, 'Messire' and P629, the partially resistant accessions JI2480 and
128 P615 and the resistant accessions P42 and P633.
129 For all experiments, germinated pea seedlings were sown in vermiculite and grown in a controlled
130 environment under a 16/8 h light-dark photoperiod at 26 ± 2 °C with 200 µmol/m^2/s of illumination.
131 Plants were watered every three days with tap water.

Pathogenicity Test. To determine the pathogenicity of each *Fop* isolates, seven days old pea seedlings were inoculated with the dip root technique as described previously[3] and maintained in the same growth condition as above. Disease was then evaluated every three days by estimating the percentage of leaves with symptoms and the Area under the disease progression curve (AUDPC).[3] Five plants were used per accessions and each experiment was repeated twice independently.

Extraction and Purification of Fusaric and 9,10-Dehydrofusaric Acids from Fungal Culture Filtrates. Lyophilized *Fop* culture filtrates (200 mL) were re-dissolved in 1/10 of the initial volume with distilled water. The solutions were adjusted to pH 2.5 with 1M HCl and extracted with EtOAc (3 X 20 mL). The acidic organic extracts were combined, dried (Na_2SO_4) and evaporated under reduced pressure yielding a brown oily residues (38.9, 101.7, 36.4, 42.7, 29.5 mg for F42, F69, *Fop1*,Arg3 and Pt1 respectively). The five acidic organic extracts were analyzed by TLC on silica gel [eluent EtOAc-MeOH-H_2O (8.5:2:1, v/v/v) and on reverse phase [eluent CH_3CN-H_2O (1:1, v/v)], in comparison with authentic standard samples of **1**, **2**, and their methyl esters. To confirm the presence of **1** and **2**, the residue of organic extract of F42, was purified by TLC eluted with CH_3CN-H_2O (1:1, v/v) yielding two pure solid compounds **1**, R_f 0.50, eluent EtOAc-MeOH-H_2O (8.5:2:1, v/v/v), R_f 0.51 eluent CH_3CN-H_2O (1:1, v/v) and **2,** R_f 0.40, eluent EtOAc-MeOH-H_2O (8.5:2:1, v/v/v), R_f 0.58, eluent CH_3CN-H_2O (1:1, v/v) which were identified as fusaric acid and 9,10-dehydrofusaric acid as described below.

Fusaric acid (**1**). IR, UV, and ^{1}H NMR spectra were very similar to data reported[15] ESIMS (+) *m/z*: 381 $[2M+Na]^+$, 202 $[M+Na]^+$: ESIMS (-) *m/z*: 178 $[M-H]^-$; APCIMS (+) *m/z*: 180 $[M+H]^+$.

Dehydrofusaric acid (**2**). IR, UV, and ^{1}H NMR spectra were very similar to data reported.[15] ESIMS (+) *m/z*: 200 $[M+Na]^+$; ESIMS (-) *m/z*: 176 $[M-H]^-$.

HPLC Analysis of Acidic Organic Extracts. A method previously reported[16] was optimized and used for analysis. The mobile phases employed were MeOH (eluent A) and 1%

158 K_2HPO_4 adjusted to pH 7.35 with concentrated H_3PO_4 (eluent B). Elution was initially with A:B
159 (50:50) which was transformed using a linear gradient over 20 min to A:B (75:25); the initial
160 conditions were restored using a linear gradient over 5 min, and the column was re-equilibrated
161 under these conditions for 10 min before the next run was initiated. The flow rate was 1 mL/min and
162 20 μL aliquots of the samples were injected for analysis. Detection was performed at 268 nm,
163 corresponding to the maximum of absorption of **1** and **2**. The HPLC calibration curves for
164 quantitative determination of **1** and **2** were performed with absolute amounts of standards dissolved
165 in MeOH in the range between 0.2 and 20 μg for each compound, in triplicate for each
166 concentration. HPLC linear regression curves (absolute amount against chromatographic peak area)
167 for **1** and **2**, were obtained based on weighted values calculated from seven amounts of the
168 standards in the above range. The samples were dissolved in MeOH, passed through disposable
169 filters and aliquots (20 μL) were injected into the HPLC instrument. Each sample was assayed in
170 triplicate. The quantitative determination of **1** and **2** was calculated interpolating the mean area of
171 their chromatographic peaks with the data from the calibration curves.
172
173 **Recovery Studies**. Recovery studies were performed using the best producer isolate F69.
174 Pure fusaric and 9,10-dehydrofusaric acids were added to the culture filtrate from 0.3 to 2.0 mg/L.
175 The samples were prepared as described above and the extracts analysed by HPLC to determine
176 recovery. Three replicate injections were performed for each concentration. The recovery
177 throughout the range of concentration was higher than 96±2%.
178
179 **Biological assays.**
180 *Leaf Absorption Assay.* The toxicity of the culture filtrate of *Fop* race 2 isolate F42 was assayed by
181 incubating fully expanded leaves in a F42 culture filtrate. For this, the leaf petiole was immersed
182 into an Eppendorf tube containing 1 mL of one week old fungal culture filtrate before or after

autoclaving at 121 ºC for 20 min and incubated at room temperature for 24 and 48 h. Four fully developed leaves were used for each pea accessions.

*Leaf Puncture Assay***.** A leaf-puncture bioassay on pea leaves was performed to evaluate the toxic effect of culture filtrates from all *Fop* race 2 isolate tested. Fully expanded leaves from pea plants were placed on Petri dishes containing water-agar medium and punctured by a sterile needle on the upper surface. Droplets (10 µL) of the culture filtrate or corresponding organic extract in 1% MeOH were applied on the wounded leaves at 2 mg/mL. Plates were then incubated at room temperature under darkness. After 3 days of incubation, the area (mm^2) of the necrotic lesions was measured. Droplets of a pure standard of FA and DFA at 2 mg/mL were used as positive control reactions while droplets of sterile water, Czapek Dox medium or 1% MeOH were used as negative control. The experiments were performed with four replicates for each treatment.

Statistical analysis. Analyses of variance (ANOVA) were carried out for phytotoxicity records, with the different tested solutions and metabolites as fixed factors of their respective bioassays. One way ANOVA was also performed to test the significance of aggressiveness differences between each strains. Phytotoxicity and percentage of symptoms values were transformed using the square root transformation in order to increase the normality of their distribution. Whenever the ANOVA test was statistically significant ($p \leq 0.05$), a Duncan's multiple range test assessing the differences of the means between each treatment was performed. All statistical analyses were performed using Genstat release 11.1 software (VSN International Ltd., Hemel Hempstead, UK).

RESULTS AND DISCUSSION

F. oxysporum species complex is composed of many species very closely related morphologically which make them difficult to identify. To ensure that all fungal strains corresponded to isolates of the race 2 of *F. oxysporum* f. sp. *pisi* these isolates were characterized at morphological and molecular levels before further analysis. As expected, all fungal strains showed

morphological characteristics of *F. oxysporum* complex species. Amplification of ITS and EF-alpha gave sequences ranging from 544-545 bp and 710-714 bp long respectively according to the isolate (Table S1). These sequences showed between 99-100% identity to *F. oxysporum* sequences from different formae speciales including ff. spp. *pisi, ciceri, medicaginis* and *lycopersici* according to the BLAST comparison performed. In addition they share 99.8-100% and 98.6-100% identity to the reference *Fop* race 2 strain F42 respectively (Table S1). This clearly identified them as *F. oxysporum* although it was not possible to identify the ff. spp. to which they belong by these methods, hampered by the potential polyphyletic origin of the isolates and potential horizontal transfer of host-specificity genes.[20] Nevertheless, the *in planta* pathogenicity test indicated that they corresponded to *F. oxysoprum* f. sp. *pisi*. Indeed, all these strains were pathogenic on the susceptible pea cultivar 'Messire' (Figure 2). Slight differences in aggressiveness were observed between isolates as determined by their AUDPC value ($p < 0.001$). The strain F69 was the most aggressive reaching AUDPC values of 2388 while the isolates *Fop1* and Arg3 were the least aggressive reaching AUDPC of only 1678 and 1778.5 respectively (Figure 2A). This difference was rooted to a faster disease development induced by the strain F69 that lead to complete plant death as early as 12 days post inoculation (dpi) while the Arg3 and *Fop 1* strains required 25 days to induce a similar effect (Figure 2B). This was confirmed by the ANOVA that revealed significant differences between isolates only up to 15 days post inoculation ($p < 0.001$) while at later stages the differences were not significant ($p > 0.5$). In addition, the inoculation of a set of differential lines[1, 3] allowed assigning the new isolates Arg3 and Pt1 to the race 2 of *Fop* since accession response to these isolates followed a similar pattern as for the reference race 2 strain F42. Indeed, no or only mild symptoms were detected on the race 2 resistant accessions while the susceptible accessions 'Dark Skin perfection', P629 and 'Messire' developed characteristic wilt symptoms (Table 1). By contrast, *Fop*1 showed *near wilt* (leaf yellowing) symptoms on most accessions which impeded a clear classification of this strain to a specific race (data not shown). However, the very close molecular relationship between this isolate and the Algerian (Arg3) isolate as shown by the analysis

of EF-alpha sequence (Table S1) together with the *near wilt* symptoms detected would suggest that this isolate also belongs to the race 2 of *Fop.*

To characterize further the mechanisms of pathogenicity of *Fop* race 2 isolates that remains largely unknown, the effect of one week old *Fop* culture filtrates was evaluated on pea leaves. Incubation of pea leaves in F42 culture filtrate for 24 h induced leaf withering followed by leaf discoloration that initiate at the central vein and progress to cover the whole leaf and petiole as early as 48 h of incubation (Figure 3A). Similar effects were detected on all pea accessions tested including on resistant accessions (Figure 3B). The leaf puncture assay also showed a progressive spreading of necrosis and leaf discoloration after treatment with the fungal culture filtrates while control remained symptomless (Figure 3C). A broad effect of *F. oxysporum* culture filtrates was previously detected for *F. oxysporum* f. sp. *orthoceras* and *F. oxysporum* f. sp. *albedinis* which extract induced wilting on both host and non-host species.[25, 26] This supports the presence of a non-specific toxin within these filtrates. In addition it indicated that resistance of the pea accessions tested was not based on toxin detoxification. This was also detected in *F. oxysporum* ff. spp. *melonis*[27] and *cubense*.[28] However, in the present study, only three resistant *Pisum* spp. accessions have been tested. Thus, it may still be possible that the resistance mechanisms of other resistant pea accessions is based on toxin detoxification as detected previously in near-isogenic lines of tomato differing in their susceptibility to *F. oxysporum* f. sp. *lycopersici*.[29] Interestingly, organic extracts of the culture filtrates from all *Fop* race 2 isolates tested were able to induce leaf necrosis on 'Messire' leaves when evaluated with the leaf puncture assay after 3 days of incubation (Figure 4A). However, difference in lesion size could be detected between the strains ($p < 0.001$) in agreement with the difference in aggressiveness of these strains on this pea cultivar (Figure 4B). The lesion size ranged from 16.8 mm^2 for Arg3 to 42.4 and 58.4 mm^2 for F42 and F69 culture filtrate respectively (Figure 4B).

Various pathogenicity factors including cell-wall degrading enzymes, phytoalexin-detoxifying enzymes and toxins have been shown to mediate *F. oxysporum* virulence.[4] In contrast to

a previous study that showed the complete loss of toxic activity after autoclaving culture filtrates,[29] autoclaving only slightly attenuated the toxicity of the filtrate on leaf absorption and puncture assays (Figure 3C). This ruled out that the toxicity is due to proteins and suggest the presence of heat-stable metabolites as found for other isolates of *Fop*[30] and human pathogenic strains of *F. oxysporum*.[31]

To identify the heat-stable metabolite(s) responsible for the toxicity of *Fop* race 2 culture filtrates, the organic extract of culture filtrates obtained from the isolate F42 was fractionated and further analyzed. TLC analysis of these fractions showed that this strain produced two toxins, identified as fusaric acid, **1**, and dehydrofusaric acid, **2**, by comparison with standard samples. Their corresponding methyl esters were not detected. These results were confirmed by purification of **1** and **2** from this organic extract. The ^{1}H NMR spectrum was very similar to those previously reported.[15] The data obtained from ESI MS spectra further supported the isolation of **1** and **2**. The ESI MS recording in positive ion mode showed the sodiated dimeric form $[2M+Na]^+$ and the sodium cluster $[M+Na]^+$ at *m/z* 381 and 202, respectively. In APCI MS it showed the pseudomolecular ion $[M+H]^+$ at *m/z* 180. When the ESI MS was recorded in negative ion mode, it showed the pseudomolecular ion $[M-H]^-$ at 178. The ESI MS spectrum of **2** recorded in positive and negative ion mode showed the sodium cluster $[M+Na]^+$ and the pseudomolecular ion $[M-H]^-$ at *m/z* 200 and 176, respectively. Further studies indicated that all *Fop* race 2 isolates investigated produced **1** and **2** but not their related methyl esters. Both toxins have been already identified from cultures of *F. oxysporum* pathogenic to the parasitic plants *Striga hermonthica*.[16, 32, 33] However, it is the first time that **2** is reported from *F. oxysporum* isolates pathogenic to crops. Other toxins were not found in any of these isolates. Production of toxins showed large qualitative and quantitative variation according to growth condition including growth medium, temperature and ambient pH among others.[16, 25, 34] Thus, it could not be ruled out that these strains may produce additional toxins as described for other *F. oxysporum* strains.[11-14]

Testing these acids with the leaf puncture assay indicated that both **1** and **2** possessed phytotoxic activity and induced necrotic lesion similar to that induced by the F42 culture filtrates (Figure 5B). Comparison of lesion size indicated that **1** (55.8 mm^2) was 2.5 time more phytotoxic than **2** (22.6 mm^2). Many studies demonstrated the function of **1** in pathogenicity, although in several instance no correlation was detected between the concentration of **1** and fungal virulence.[5, 7, 35] **1** has been shown to alter membrane permeability, inhibit O_2 uptake and ATP synthesis, decrease mitochondrial activity, transpiration rate and stomatal conductance and induce cell depolarization[7, 10] suggesting that **1** participates in *F. oxysporum* pathogenicity by decreasing plant cell viability.[7] By contrast, nearly nothing is known about the action of **2**.[15] This compound was previously shown to induce tomato leaves chlorosis and inhibit root elongation to a similar extent as **1**.[15] Here we demonstrated that dehydrofusaric acid was also phytotoxic on pea leaves (Figure 4) indicating that it may also contribute to the pathogenicity of *F. oxysporum*.

To confirm the function of **1** and **2** in *Fop* race 2 pathogenicity, we quantitated the production of both acids by all *Fop* race 2 isolates tested and related it to their level of aggressiveness. For the quantitation, we slightly modified a previously reported HPLC method.[16] The characteristics of the calibration curves, the absolute range and the detection limits (LOD) of **1** and **2** are summarized in Table S2. Regression analysis suggests that the calibration curves are linear. A representative HPLC chromatogram of the ethyl acetate extract of the culture filtrates of *Fop* F42 is presented in Figure 5A. The metabolite chromatographic peaks (*a*) and (*b*) in the sample was coincident to the 7.80 min and 5.20 min retention times of **1** and **2** standards (Figure 5A). The retention times were highly reproducible, varying less than 0.50 min. For all strains matrix substances absorbing at 268 nm were eluted within the first 20 minutes. **1** could be quantitatively and reproducibly detected from 0.5 μg, and **2** from 0.2 μg, with lower amounts having poor reproducibility.

Although **1** and **2** were secreted by all isolates of *Fop* race 2 tested, this method allowed detecting quantitative differences between these isolates ($p < 0.001$) for both toxins. Of the strain tested, F69 produced the highest amount of **1** and **2** (258.49 and 243.36 mg/L, respectively) whereas the strains

312 Arg 3 and *Fop1* produced the least **1** (9.94 mg/L) and **2** (21.44 mg/L) respectively (Figure 6). The
313 capacity to produce and secrete **1** was significantly and positively correlated with the leaf lesion
314 size (r^2 = 0.83; Figure 7A) and slightly correlated with *in planta* pathogenicity (r^2 = 0.66; Figure
315 7B). Production of **2** was significantly correlated with *in planta* pathogenicity (r^2 = 0.8; Figure 7D)
316 and to a lesser extent with lesion size (r^2 = 0.66; Figure 7C). Interestingly, the total toxin production
317 was significantly correlated with both virulence parameters (r^2 = 0.78 and 0.76 for leaf lesion size
318 and *in planta* pathogenicity respectively) (Figures. 7E and F). This reinforced the important role of
319 **1** in *F. oxysporum* pathogenicity as it was already demonstrated for *F. oxysporum* ff. spp.
320 *carthami*,[36] *lycopersici*,[37, 38] *melonis*[39] and *gladioli*[40] between others. It also demonstrated that **2** is
321 an important pathogenicity factor acting in synergy with **1** which was not described before for *F.*
322 *oxysporum* (Figure 7).

323 In conclusion, it has been demonstrated that *Fop* race 2 isolates produced mainly two toxins
324 that were identified as fusaric and dehydrofusaric acids. Both toxins showed high phytotoxic
325 activity on pea when tested on whole leaves and leaf puncture assay. In addition, the amount of both
326 toxins within culture filtrates correlated with strain aggressiveness which indicates a role of these
327 toxins during *Fop* pathogenicity. Although the importance of fusaric acid in the pathogenicity of *F.*
328 *oxysporum* is still under debate, the results obtained in the present study strongly indicate that toxin
329 production is an important determinant of *Fop* race 2 pathogenicity**.**

330

331 **ASSOCIATED CONTENT**

332 **Supporting Information**

333 Table S1 and Table S3. This material is available free of charge via the Internet at
334 http://pubs.acs.org.
335

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Notes

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466 **FIGURE LEGEND**

467 **Figure 1.** Structures of fusaric acid, **1**, and 9,10-dehydrofusaric acid, **2**.

468 **Figure 2**. Pathogenicity of the *F. oxysporum* f. sp. *pisi* race 2 isolates on the susceptible pea cultivar
469 'Messire'. **A**, evolution of *Fusarium* wilt symptoms on the susceptible pea cultivar 'Messire' induced
470 by the *Fop* race 2 isolates F42 (●), F69 (■), Arg3 (○), Pt1 (△) and *Fop1* (▲) respectively, compared
471 to control plants treated with water (□). Disease progression was estimated as percentage of leaf
472 with symptoms over time. **B**, Comparison of AUDPC values calculated from the periodic
473 assessment of *Fusarium* wilt symptoms development. Different letters between each histograms
474 indicates significant difference between values according to Duncan Multiple Range Test at α =
475 0.05.Vertical Bars are standard error for n= 5.

476 **Figure 3.** Effect of the culture filtrates of *F. oxysporum* f. sp. *pisi* race 2 isolate F42 on pea leaves.
477 **A**, progression of wilting symptoms induced on the partially resistant accession JI2480 by the
478 culture filtrates after 24 and 48h of treatment (F) compare to control with sterile water (C). **B**, Effect
479 of the culture filtrates after 24h of treatment on leaves of seven pea accessions differing in their
480 susceptibility to *Fop* race 2. **C**, Comparison of the leave response of the resistant pea accession P42
481 to culture filtrates, autoclaved culture filtrates, sterile Czapek Dox medium and sterile water
482 evaluated with the leaf absorption and the leaf puncture assays.

483 **Figure 4.** Effect of culture filtrates of the different *F. oxysporum* f. sp. *pisi* race 2 isolates on leaves
484 of the susceptible pea cultivar 'Messire'. **A**, Comparison of 'Messire' leaves response to culture
485 filtrates of each *Fop* race 2 isolates or sterile Czapek Dox medium (Control). **B**, Comparison of the
486 lesion size induced by each isolate or by sterile Czapek Dox medium (C). Different letters between
487 each histograms indicates significant difference between value according to Duncan Multiple Range
488 Test at α = 0.05.Vertical bars represent standard errors for n= 4.

489 **Figure 5.** Characteristics of the main toxins produced by the *F. oxysporum* f. sp. *pisi* race 2 isolates.
490 **A**, HPLC profile of the organic extract of the *Fop* race 2 isolate F42 (left) and fusaric and 9,10-

491 dehydrofusaric acids (**1** and **2**) standards (right). **B**, Comparison of the leaf response of the
492 susceptible pea cultivar 'Messire' to droplets of pure **1** and **2.**

493 **Figure 6.** Quantitation of the amount of **1** and **2** produced by the different isolates of *F. oxysporum*
494 f. sp. *pisi* race 2. The histograms show the cumulative production of **1** (black column) and **2** (white
495 column) for each *Fop* race 2 isolates. Vertical bars are standard error for n=3.

496 **Figure 7.** Relationship between the toxins production and phytotoxicity. The graphics represent the
497 linear correlation calculated for **1** (A, B), **2** (C, D) and the sum of both toxins (E, F) production by
498 each *Fop* race 2 isolates with the lesion size induced by their corresponding culture filtrate (A, C, E)
499 or their overal aggressiveness on the susceptible pea cutivar 'Messire' estimated by their AUDPC
500 values (B, D, F). Horizontal bar are standard error for lesion size area or AUDPC values with n= 4
501 and n= 5 respectively while vertical bars are standard error bars of toxin content calculated with
502 n=3.

503

Tables

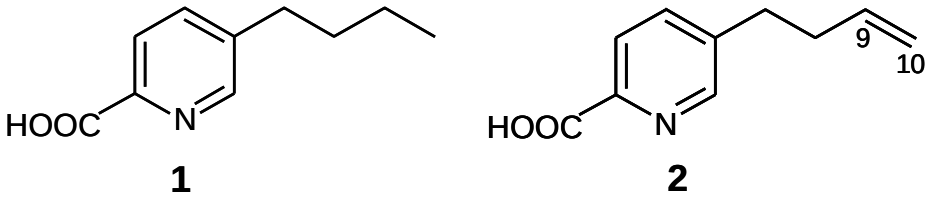
Table 1. List of the Pea Differential Lines for *F. oxysporum* f. sp. *pisi* and their Susceptibility Response to the New *Fop* Isolates.

Accessions	Expected[a]				Observed[b]		
	R1	R2	R5	R6	F42	Arg3	Pt1
'Dark Skin Perfection'	R	S	S	S	S	S	S
'Mini'	S	R	S	S	PR	PR	R
'New Era'	R	R	S	S	nd	R	R
'New Season'	R	R	S	R	R	R	R
'74SN5'	R	R	R	R	R	R	R
P629	R	S	nd	nd	S	S	S
JI1412	R	R	nd	nd	R	R	R
'Messire'	R	S	nd	nd	S	S	S

[a] Accessions response to each *Fop* races as described in Kraft and Pfleger (2001) and Bani et al. (2013). R stand for resistant reaction and S susceptible.

[b] Accessions response to the inoculation with the reference R2 strain F42 and the isolates from Algeria and Portugal obtained in the present study

Figure 1.



1 **2**

Figure 2

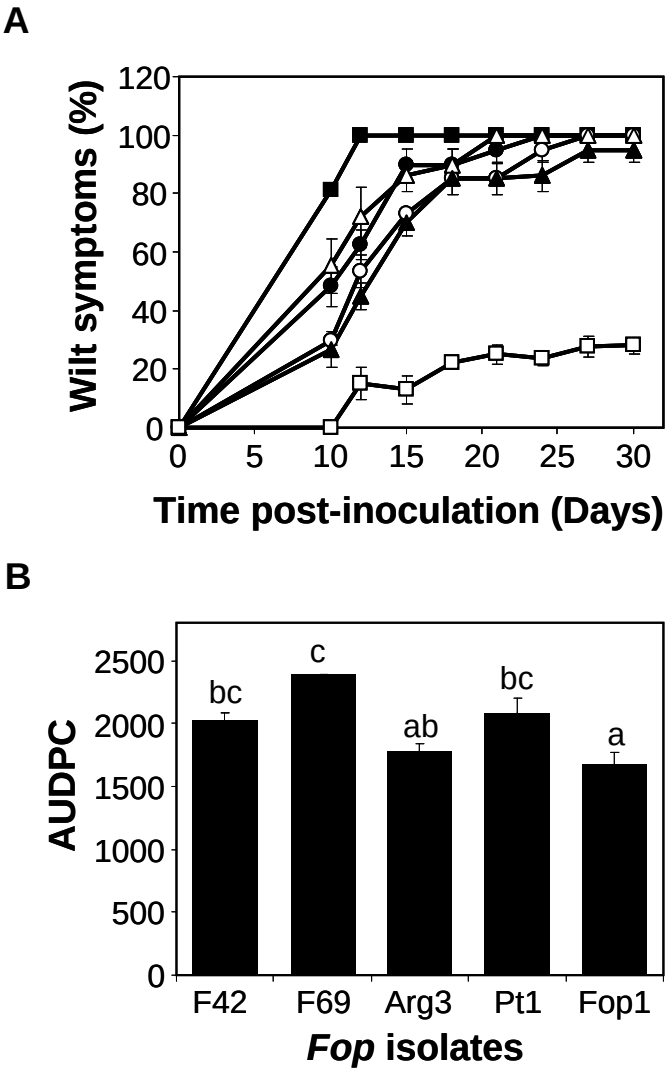

A
Wilt symptoms (%)
120
100
80
60
40
20
0
0
5
10
15
20
25
30
Time post-inoculation (Days)
B
AUDPC
2500
2000
1500
1000
500
0
bc
c
ab
bc
a
F42
F69
Arg3
Pt1
Fop1
Fop isolates

Figure 3.

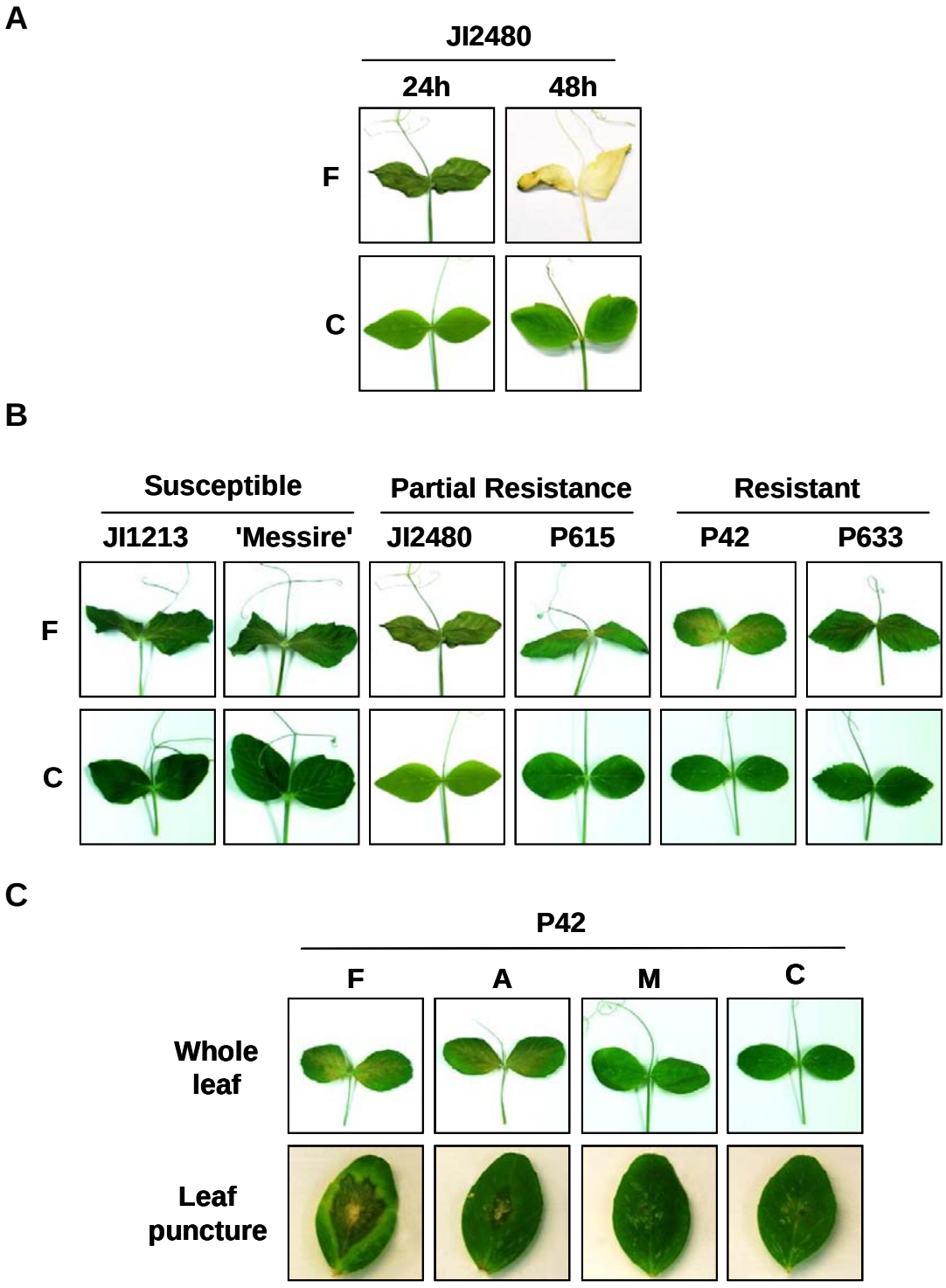
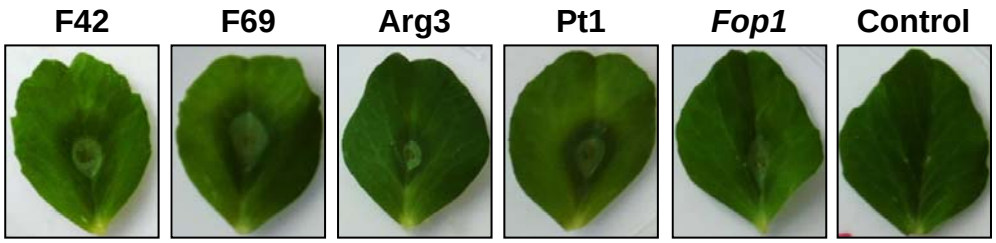

A
JI2480
24h
48h
F
C
B
Susceptible
Partial Resistance
Resistant
JI1213
'Messire'
JI2480
P615
P42
P633
F
C
C
P42
F
A
M
C
Whole leaf
Leaf puncture

Figure 4.

A


F42
F69
Arg3
Pt1
Fop1
Control

B

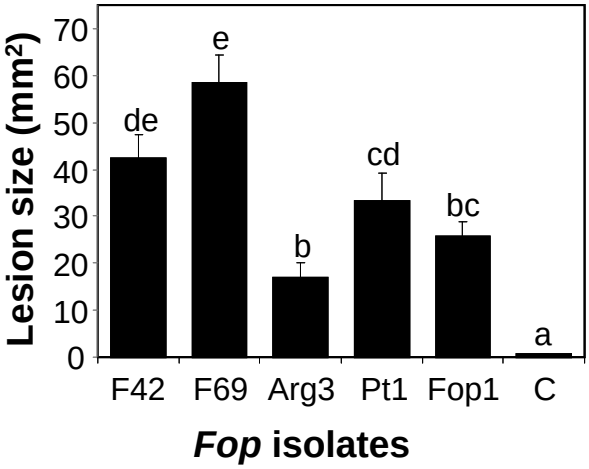

Lesion size (mm²)
70
60
50
40
30
20
10
0
de
e
b
cd
bc
a
F42
F69
Arg3
Pt1
Fop1
C
Fop isolates

Figure 5.

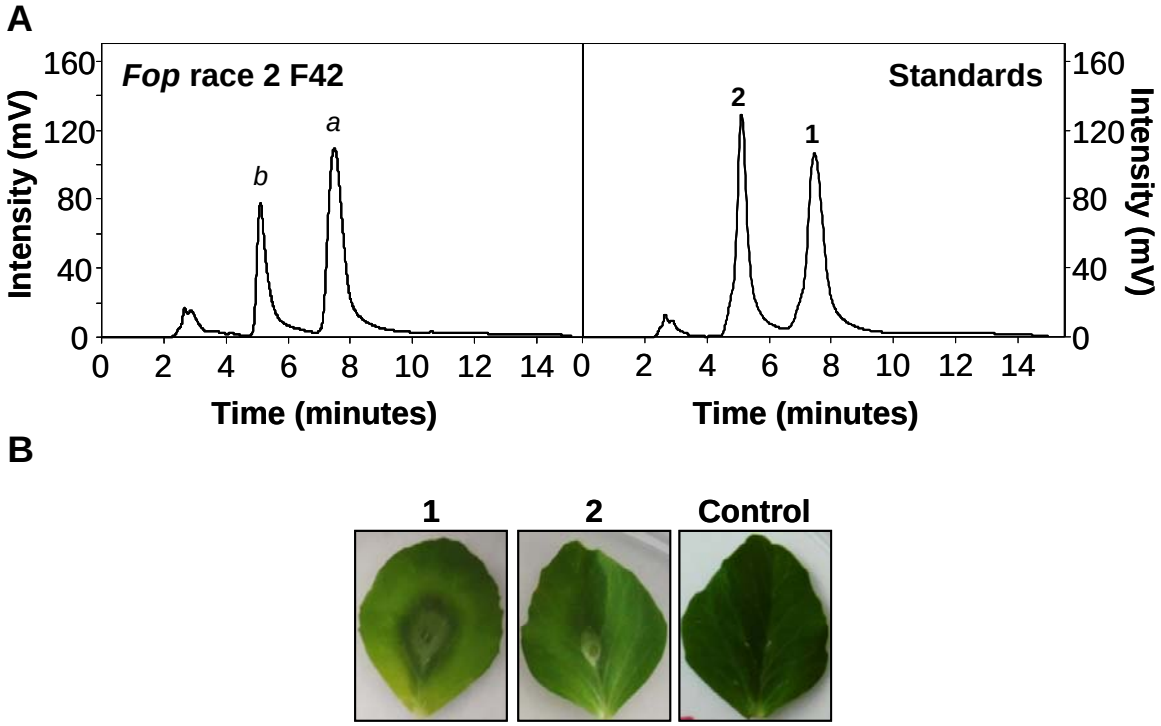

A
Fop race 2 F42
Standards
Intensity (mV)
Time (minutes)
a
b
1
2
B
1
2
Control

Figure 6.

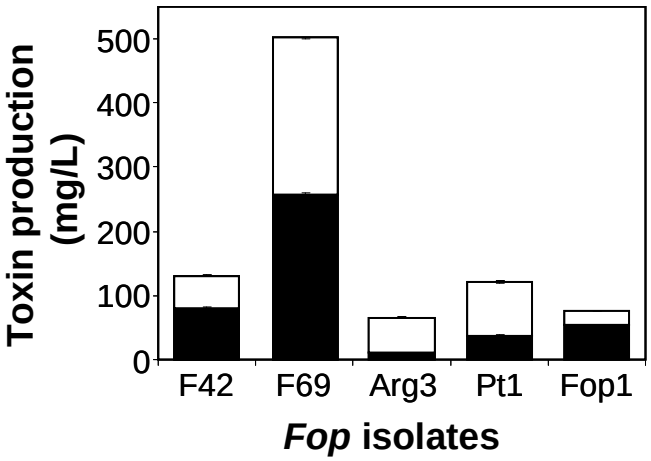
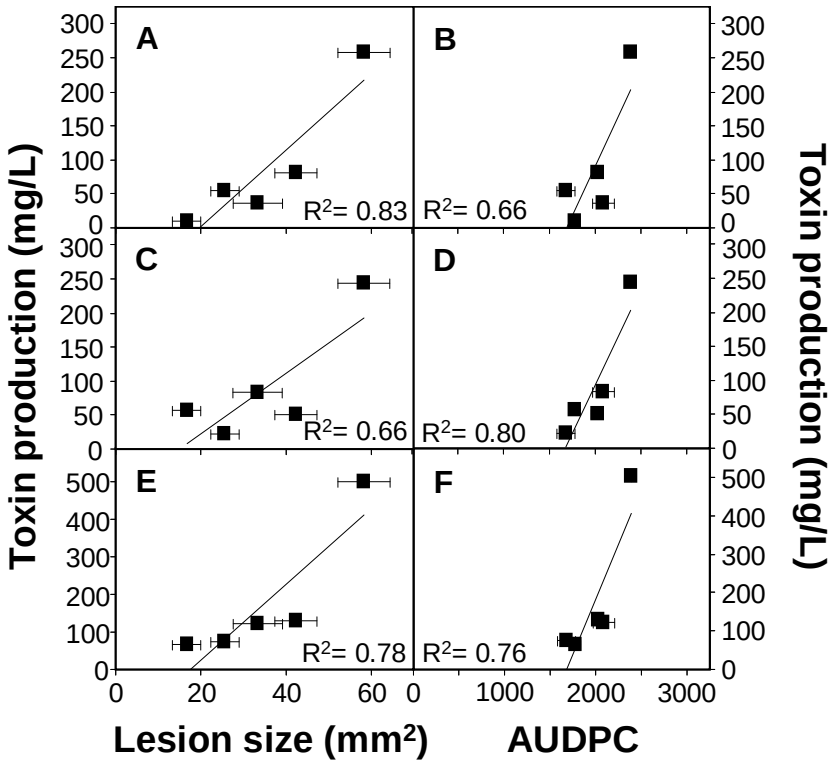

Toxin production (mg/L)
500
400
300
200
100
0
F42
F69
Arg3
Pt1
Fop1
Fop isolates

Figure 7.


A
B
C
D
E
F
R2= 0.83
R2= 0.66
R2= 0.66
R2= 0.80
R2= 0.78
R2= 0.76
Toxin production (mg/L)
Toxin production (mg/L)
Lesion size (mm2)
AUDPC

SUPPORTING INFORMATION

Identification of the Main Toxins Isolated from *Fusarium oxysporum* f. sp. *pisi* Race 2 and Their Relation with Isolates Pathogenicity

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Table S1. Comparison of ITS and EF-alpha Sequence of the *Fop* Isolates with the Race 2 Reference Strain of *Fop* F42.

Fop isolates	ITS			EF-alpha		
	Genbank number	Length (bp)	Identity (%)	Genbank number	Length (bp)	Identity (%)
F42	KF913723	544	100	KF913728	713	100
F69	KF913724	544	100	KF913729	713	100
Arg3	KF913725	544	99.8	KF913730	714	99.0
Pt1	KF913726	545	99.8	KF913731	712	98.6
Fop1	KF913727	544	100	KF913732	714	99.0

Table S2. Analytical Characteristics of Calibration Curve[a] for fusaric acid, **1** and dehydrofusaric acid, **2**.

Compound	R_t **(min)**	**Range (µg)**	**Slope**	**Intercept**	r^2	**Number of data point**	**LOD(µg)** [b]
1	7.80 ± 0.5 min	0.5-20	8015,9	- 1700,5	0.999	6	0.5
2	5.20 ± 0.5 min	0.2-20	6449,5	- 1243,7	0.998	7	0.2

[a]Calculated in the form y=a+bx where y=chromatographic peak area and x=µg of metabolite injected.
[b]Limit of detection