**Identification of the Main Toxins Isolated from *Fusarium oxysporum* f. sp. *pisii* 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

28 INTRODUCTION

29 *Fusarium oxysporum* f.sp. *pisi* W.C. Snyder & H.N. Hansen. (*Fop*) is an important pathogen
 30 causing vascular wilt of field pea (*Pisum sativum* L.) worldwide.¹ Four different races of *Fop*, races
 31 1, 2, 5 and 6 have been described.² Races 1 and 2 have been reported in every country where peas
 32 are grown, while races 5 and 6 are, to date, only important in western Washington State.² Plants
 33 infected with race 2 are most often scattered throughout the field rather than being concentrated in
 34 specific areas as observed with the other races, which is described as *near wilt*.¹ Resistance to *Fop*
 35 race 1, 5 and 6 is conferred by single dominant gene while resistance to race 2 have been recently
 36 shown to be quantitative.^{2, 3}

37 Several virulence factors have been reported to play a role at different stages of the infection
 38 process to induce disease and counteract the plant defence reaction in several formae speciales (ff.
 39 spp). of *Fusarium oxysporum*.⁴ However, these studies have not targeted the virulence factors of
 40 *Fop*. One of the best studied virulence factors is the fusaric acid, **1** (Figure 1). Fusaric acid is a non-
 41 specific toxin produced by many *Fusarium* species.^{5, 6} At high concentration it induces many
 42 physiological responses in plant cells including alteration of cell growth, mitochondrial activity and
 43 membrane permeability while at lower concentration it can trigger plant defence reactions and
 44 programmed cell death.^{7, 8} Fusaric acid was also shown to induce wilt symptoms on pepper and
 45 cucumber.^{9, 10} Thus **1** is considered to participate in *F. oxysporum* pathogenicity by reducing plant
 46 cell viability. However, **1** was also detected within plant tissue colonized by non-pathogenic isolates
 47 which questions the exact importance of **1** during the infection process.⁷ Thus, the role of **1** in *F.*
 48 *oxysporum* pathogenicity is still under debate. Apart from fusaric acid, some *F. oxysporum* isolates
 49 can produce additional toxins such as beauvericin, enniantin B, bikaverin, moniliformin, fumonisin
 50 and trichothecenes¹¹⁻¹⁴ that can also contribute to their pathogenicity. Toxins produced by *Fop* and
 51 their potential function in *Fop* pathogenicity is still unknown. To improve understanding on *Fop*
 52 pathogenicity, the main toxins of several isolates of *Fop* race 2 were identified and quantitated.

54 MATERIALS AND METHODS

55 **General Experimental Procedures.** IR spectra were recorded as deposited glass film on a
56 Perkin-Elmer (Norwalk, CT) Spectrum One FT-IR spectrometer and UV spectra were measured in
57 MeCN on a Perkin-Elmer Lambda 23 UV/Vis spectrophotometer. ¹H NMR spectra were recorded
58 at 600 or 400 MHz, in CD₃OD, on Bruker (Kalsruhe, Germany) spectrometers. The same solvent
59 was used as internal standard. ESI and APCI MS spectra were recorded on an Agilent Technologies
60 (Milan, Italy) 6120 Quadrupole LC/MS instrument. Analytical and HPLC grade solvents for
61 chromatography were purchased from Carlo Erba (Milan, Italy). All other analytical grade
62 chemicals were purchased from Merck (Darmstadt, Germany). Analytical and preparative thin layer
63 chromatography (TLC) were performed on silica gel (Kieselgel 60, F₂₅₄, 0.25 and 0.5 mm
64 respectively) (Merck, Darmstadt, Germany) or reverse phase (KC18 F₂₅₄, 0.20 mm) (Whatman,
65 Maidstone, UK) plates. The spots were visualized by exposure to UV radiation (254 nm), or by
66 spraying first with 10% H₂SO₄ in MeOH and then with 5% phosphomolybdic acid in EtOH,
67 followed by heating at 110 °C for 10 min, or by exposure to iodine vapours. The HPLC system
68 (Shimadzu, Tokyo, Japan) consisted of a Series LC-10AdVP pump, FCV-10AlVP valves, SPD-
69 10AVVP spectrophotometric detector and DGU-14A degasser. The HPLC column used was 250 x
70 4.6 mm i.d.; 5 µm high-density Nucleosil 100-5 RP18 (Macherey-Nagel, Duren, Germany)
71 preceded by an in-line guard column (Alltech, Sedriano, Italy). Water was HPLC quality, purified
72 in a Milli-Q system (Millipore, Bedford, MA, USA). Disposable syringe filters, Anotop 10-0,2 µm,
73 were purchased from Whatman (Maidstone, UK). Fusaric acid was purchased from Sigma (St.
74 Louis, MO, USA)

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

77 **Purification of 9,10-dehydrofusaric acid.** 9,10-dehydrofusaric acid, **2** (Figure 1) was
78 purified from fungal culture filtrates of *Fusarium nygamai* as previously described by Capasso et
79 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 (*Fop1*) 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 previously³ 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 °C in 30% glycerol. For microconidia production, cultures were grown in potato dextrose broth (PDB) (Difco, Detroit, MI) at 28 °C 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% K₂HPO₄, 0.2% NaNO₃, 0.05% MgSO₄·7H₂O and 0.001% FeSO₄·7H₂O), were inoculated with 200 µL of fresh *Fop* microconidia at 10⁹ 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 °C 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-4¹⁹ and EF-1/EF2²⁰

106 respectively. Each 50 μ L reaction mixture contained 50 ng of template DNA, 2 units of BioTaq
 107 DNA polymerase (Bioline, London, UK), 1 $\times$ PCR buffer, 2 mM MgCl₂, 200 μ M dNTPs, and 0.3
 108 μ M 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 BLAST²¹ searches against the GenBank²² and Mycobank²³ databases to identify the most similar
 118 sequences available in the databases. Pair-wise alignments were performed with the Neddleman-
 119 Wunsch algorithm (Needle method) implemented at EMBL-EBI webserver²⁴. 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 $\pm$ 2 °C with 200 μ mol/m²/s of illumination.
 131 Plants were watered every three days with tap water.

132 **Pathogenicity Test.** To determine the pathogenicity of each *Fop* isolates, seven days old
 133 pea seedlings were inoculated with the dip root technique as described previously³ and maintained
 134 in the same growth condition as above. Disease was then evaluated every three days by estimating
 135 the percentage of leaves with symptoms and the Area under the disease progression curve
 136 (AUDPC).³ Five plants were used per accessions and each experiment was repeated twice
 137 independently.

138 **Extraction and Purification of Fusaric and 9,10-Dehydrofusaric Acids from Fungal**
 139 **Culture Filtrates.** Lyophilized *Fop* culture filtrates (200 mL) were re-dissolved in 1/10 of the
 140 initial volume with distilled water. The solutions were adjusted to pH 2.5 with 1M HCl and
 141 extracted with EtOAc (3 X 20 mL). The acidic organic extracts were combined, dried (Na₂SO₄) and
 142 evaporated under reduced pressure yielding a brown oily residues (38.9, 101.7, 36.4, 42.7, 29.5 mg
 143 for F42, F69, *FopI*, Arg3 and Pt1 respectively). The five acidic organic extracts were analyzed by
 144 TLC on silica gel [eluent EtOAc-MeOH-H₂O (8.5:2:1, v/v/v) and on reverse phase [eluent CH₃CN-
 145 H₂O (1:1, v/v)], in comparison with authentic standard samples of **1**, **2**, and their methyl esters. To
 146 confirm the presence of **1** and **2**, the residue of organic extract of F42, was purified by TLC eluted
 147 with CH₃CN-H₂O (1:1, v/v) yielding two pure solid compounds **1**, *R_f* 0.50, eluent EtOAc-MeOH-
 148 H₂O (8.5:2:1, v/v/v), *R_f* 0.51 eluent CH₃CN-H₂O (1:1, v/v) and **2**, *R_f* 0.40, eluent EtOAc-MeOH-
 149 H₂O (8.5:2:1, v/v/v), *R_f* 0.58, eluent CH₃CN-H₂O (1:1, v/v) which were identified as fusaric acid
 150 and 9,10-dehydrofusaric acid as described below.

151 *Fusaric acid (1).* IR, UV, and ¹H NMR spectra were very similar to data reported¹⁵ ESIMS
 152 (+) *m/z*: 381 [2M+Na]⁺, 202 [M+Na]⁺; ESIMS (-) *m/z*: 178 [M-H]⁻; APCIMS (+) *m/z*: 180 [M+H]⁺.

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

155

156 **HPLC Analysis of Acidic Organic Extracts.** A method previously reported¹⁶ was
 157 optimized and used for analysis. The mobile phases employed were MeOH (eluent A) and 1%

158 K₂HPO₄ adjusted to pH 7.35 with concentrated H₃PO₄ (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 °C 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 (mm²) 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).

203

204 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. oxysporum* 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, *Fop1* 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

235 of EF-alpha sequence (Table S1) together with the *near wilt* symptoms detected would suggest that
 236 this isolate also belongs to the race 2 of *Fop*.
 237 To characterize further the mechanisms of pathogenicity of *Fop* race 2 isolates that remains largely
 238 unknown, the effect of one week old *Fop* culture filtrates was evaluated on pea leaves. Incubation
 239 of pea leaves in F42 culture filtrate for 24 h induced leaf withering followed by leaf discoloration
 240 that initiate at the central vein and progress to cover the whole leaf and petiole as early as 48 h of
 241 incubation (Figure 3A). Similar effects were detected on all pea accessions tested including on
 242 resistant accessions (Figure 3B). The leaf puncture assay also showed a progressive spreading of
 243 necrosis and leaf discoloration after treatment with the fungal culture filtrates while control
 244 remained symptomless (Figure 3C). A broad effect of *F. oxysporum* culture filtrates was previously
 245 detected for *F. oxysporum* f. sp. *orthoceras* and *F. oxysporum* f. sp. *albedinis* which extract induced
 246 wilting on both host and non-host species.^{25, 26} This supports the presence of a non-specific toxin
 247 within these filtrates. In addition it indicated that resistance of the pea accessions tested was not
 248 based on toxin detoxification. This was also detected in *F. oxysporum* ff. spp. *melonis*²⁷ and
 249 *cubense*.²⁸ However, in the present study, only three resistant *Pisum* spp. accessions have been
 250 tested. Thus, it may still be possible that the resistance mechanisms of other resistant pea accessions
 251 is based on toxin detoxification as detected previously in near-isogenic lines of tomato differing in
 252 their susceptibility to *F. oxysporum* f. sp. *lycopersici*.²⁹ Interestingly, organic extracts of the culture
 253 filtrates from all *Fop* race 2 isolates tested were able to induce leaf necrosis on 'Messire' leaves
 254 when evaluated with the leaf puncture assay after 3 days of incubation (Figure 4A). However,
 255 difference in lesion size could be detected between the strains ($p < 0.001$) in agreement with the
 256 difference in aggressiveness of these strains on this pea cultivar (Figure 4B). The lesion size ranged
 257 from 16.8 mm² for Arg3 to 42.4 and 58.4 mm² for F42 and F69 culture filtrate respectively (Figure
 258 4B).

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

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

266 To identify the heat-stable metabolite(s) responsible for the toxicity of *Fop* race 2 culture
 267 filtrates, the organic extract of culture filtrates obtained from the isolate F42 was fractionated and
 268 further analyzed. TLC analysis of these fractions showed that this strain produced two toxins,
 269 identified as fusaric acid, **1**, and dehydrofusaric acid, **2**, by comparison with standard samples. Their
 270 corresponding methyl esters were not detected. These results were confirmed by purification of **1**
 271 and **2** from this organic extract. The ¹H NMR spectrum was very similar to those previously
 272 reported.¹⁵ The data obtained from ESI MS spectra further supported the isolation of **1** and **2**. The
 273 ESI MS recording in positive ion mode showed the sodiated dimeric form $[2M+Na]^+$ and the
 274 sodium cluster $[M+Na]^+$ at m/z 381 and 202, respectively. In APCI MS it showed the
 275 pseudomolecular ion $[M+H]^+$ at m/z 180. When the ESI MS was recorded in negative ion mode, it
 276 showed the pseudomolecular ion $[M-H]^-$ at 178. The ESI MS spectrum of **2** recorded in positive and
 277 negative ion mode showed the sodium cluster $[M+Na]^+$ and the pseudomolecular ion $[M-H]^-$ at m/z
 278 200 and 176, respectively. Further studies indicated that all *Fop* race 2 isolates investigated
 279 produced **1** and **2** but not their related methyl esters. Both toxins have been already identified from
 280 cultures of *F. oxysporum* pathogenic to the parasitic plants *Striga hermonthica*.^{16, 32, 33} However, it
 281 is the first time that **2** is reported from *F. oxysporum* isolates pathogenic to crops. Other toxins were
 282 not found in any of these isolates. Production of toxins showed large qualitative and quantitative
 283 variation according to growth condition including growth medium, temperature and ambient pH
 284 among others.^{16, 25, 34} Thus, it could not be ruled out that these strains may produce additional toxins
 285 as described for other *F. oxysporum* strains.¹¹⁻¹⁴

286 Testing these acids with the leaf puncture assay indicated that both **1** and **2** possessed
 287 phytotoxic activity and induced necrotic lesion similar to that induced by the F42 culture filtrates
 288 (Figure 5B). Comparison of lesion size indicated that **1** (55.8 mm²) was 2.5 time more phytotoxic
 289 than **2** (22.6 mm²). Many studies demonstrated the function of **1** in pathogenicity, although in
 290 several instance no correlation was detected between the concentration of **1** and fungal virulence.^{5, 7,}
 291 ³⁵ **1** has been shown to alter membrane permeability, inhibit O₂ uptake and ATP synthesis, decrease
 292 mitochondrial activity, transpiration rate and stomatal conductance and induce cell depolarization^{7,}
 293 ¹⁰ suggesting that **1** participates in *F. oxysporum* pathogenicity by decreasing plant cell viability.⁷
 294 By contrast, nearly nothing is known about the action of **2**.¹⁵ This compound was previously shown
 295 to induce tomato leaves chlorosis and inhibit root elongation to a similar extent as **1**.¹⁵ Here we
 296 demonstrated that dehydrofusaric acid was also phytotoxic on pea leaves (Figure 4) indicating that
 297 it may also contribute to the pathogenicity of *F. oxysporum*.

298 To confirm the function of **1** and **2** in *Fop* race 2 pathogenicity, we quantitated the production
 299 of both acids by all *Fop* race 2 isolates tested and related it to their level of aggressiveness. For the
 300 quantitation, we slightly modified a previously reported HPLC method.¹⁶ The characteristics of the
 301 calibration curves, the absolute range and the detection limits (LOD) of **1** and **2** are summarized in
 302 Table S2. Regression analysis suggests that the calibration curves are linear. A representative HPLC
 303 chromatogram of the ethyl acetate extract of the culture filtrates of *Fop* F42 is presented in Figure
 304 5A. The metabolite chromatographic peaks (a) and (b) in the sample was coincident to the 7.80 min
 305 and 5.20 min retention times of **1** and **2** standards (Figure 5A). The retention times were highly
 306 reproducible, varying less than 0.50 min. For all strains matrix substances absorbing at 268 nm were
 307 eluted within the first 20 minutes. **1** could be quantitatively and reproducibly detected from 0.5 µg,
 308 and **2** from 0.2 µg, with lower amounts having poor reproducibility.

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

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

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

ASSOCIATED CONTENT

Supporting Information

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

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346

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353

354 **REFERENCES**

- 355 (1) Kraft, J.M.; Pflieger, F.L. *Compendium of pea diseases and pests*, edition n. 2; APS Press:
 356 St Paul, Minnesota, USA: 2001
- 357 (2) Infantino, A.; Kharrat, M.; Riccioni, L.; Coyne, C.J.; Mcphee, K.E.; Grunwald, N.J.
 358 Screening techniques and sources of resistance to root diseases in cool season food legumes.
 359 *Euphytica* **2006**, *147*, 201-221.
- 360 (3) Bani, M.; Rubiales, D.; Risipail, N. A detailed evaluation method to identify sources of
 361 quantitative resistance to *Fusarium oxysporum* f. sp. *pisi* race 2 within a *Pisum* spp. germplasm
 362 collection. *Plant Pathol* **2012**, *61*, 532-542.
- 363 (4) Roncero, M.I.G.; Hera, C.; Ruiz-Rubio, M.; García Maceira, F.I.; Madrid M. P., Caracuel,
 364 Z.; Calero, F.; Delgado-Jarana, J.; Roldán-Rodríguez, R.; Martínez-Rocha, A.L.; Velasco, C.; Roa,

- 365 J.; Martín-Urdiroz, M.; Córdoba, D.; Di Pietro, A. *Fusarium* as a model for studying virulence in
366 soilborne plant pathogens. *Physiol. Mol. Plant Pathol.* **2003**, *62*, 87-98.
- 367 (5) Bacon, C.W.; Porter, J.K.; Norred, W.P.; Leslie, J.F. Production of fusaric acid by
368 *Fusarium* species. *Appl. Environ. Microbiol.* **1996**, *62*, 4039-4043.
- 369 (6) Abouzeid, M.A.; Boari, A.; Zonno, M.C.; Vurro, M.; Evidente A. Toxicity profiles of
370 potential biocontrol agents of *Orobanche ramosa*. *Weed Sci.* **2004**, *52*, 326-332.
- 371 (7) Bouizgarne, B.; El-Maarouf-Bouteau, H.; Frankart, C.; Reboutier, D.; Madiona, K.;
372 Pennarun, A.M.; Monesteiz, M.; Trouviere, J.; Amiar, Z.; Briand, J.; Brault, M.; Rona, J.P.;
373 Ouhdoch, Y.; El Hadrami, I.; Bouteau, F. Early physiological responses of *Arabidopsis thaliana*
374 cells to fusaric acid: toxic and signalling effects. *New Phytol.* **2006**, *169*, 209-218.
- 375 (8) Jiao, J.; Zhou, B.; Zhu, X.; Gao, Z.; Liang, Y. Fusaric acid induction of programmed cell
376 death modulated through nitric oxide signalling in tobacco suspension cells. *Planta* **2013**, *238*, 727-
377 737.
- 378 (9) Sarhan, A.R.T.; Hegazi, A.M.A. Effect of nitrogen, phosphorus and potassium on
379 sensitivity of pepper plants to *Fusarium* wilt toxin. *Acta Phytopathol. Hun.* **1988**, *23*, 57-60.
- 380 (10) Wang, M.; Xiong, Y.; Ling, N.; Feng, X.; Zhong, Z.; Shen, Q.; Guo, S. Detection of the
381 dynamic response of cucumber leaves to fusaric acid using thermal imaging. *Plant Physiol.*
382 *Biochem.* **2012**, *66*, 68-76.
- 383 (11) Mirocha, C.J.; Abbas, H.K.; Kommedahl, T.; Jarvis, B.B. Mycotoxin production by
384 *Fusarium oxysporum* and *Fusarium sporotrichioides* isolated from *Baccharis* spp. from Brazil.
385 *Appl. Environ. Microbiol.* **1989**, *55*, 254-255.
- 386 (12) Moretti, A.; Belisario, A.; Tafuri, A.; Ritieni, A.; Corazza, L.; Logrieco, A. Production of
387 beauvericin by different races of *Fusarium oxysporum* f. sp. *melonis*, the *Fusarium* wilt agent of
388 muskmelon. *Eur. J. Plant Pathol.* **2002**, *108*, 661-666.

- 389 (13) Son, S.W.; Kim, H.Y.; Choi, G.J.; Lim, H.K.; Jang, K.S.; Lee, S.O.; Lee, S.; Sung, N.D.;
 390 Kim, J.-C. Bikaverin and fusaric acid from *Fusarium oxysporum* show antioomycete activity
 391 against *Phytophthora infestans*. *J. Appl. Microbiol.* **2008**, *104*, 692-698.
- 392 (14) Irzykowska, L.; Bocianowski, J.; Waskiewicz, A.; Weber, Z.; Karolewski, Z.; Golinski, P.;
 393 Kostecki, M.; Irzykowski, W. Genetic variation of *Fusarium oxysporum* isolates forming fumonisin
 394 B-1 and moniliformin. *J. Appl. Gen.* **2012**, *53*, 237-247.
- 395 (15) Capasso, R.; Evidente, A.; Cutignano, A.; Vurro, M.; Zonno, M.C.; Bottalico, A. Fusaric
 396 and 9,10-dehydrofusaric acids and their methyl esters from *Fusarium nygamai*. *Phytochemistry*
 397 **1996**, *41*, 1035-1039.
- 398 (16) Amalfitano, C.; Pengue, R.; Andolfi, A.; Vurro, M.; Zonno, M.C.; Evidente, A. HPLC
 399 analysis of fusaric acid, 9,10-dehydrofusaric acid and their methyl esters, toxic metabolites from
 400 weed pathogenic *Fusarium* species. *Phytochem. Anal.* **2002**, *13*, 277-282.
- 401 (17) Di Pietro, A.; Roncero, M.I.G. Cloning, expression, and role in pathogenicity of *pgl*
 402 encoding the major extracellular endopolygalacturonase of the vascular wilt pathogen *Fusarium*
 403 *oxysporum*. *Mol. Plant Microbe Interact.* **1998**, *11*, 91-98.
- 404 (18) Raeder, U.; Broda, P. Rapid preparation of DNA from filamentous fungi. *Lett. Appl.*
 405 *Microbiol.* **1985**, *1*, 17-20.
- 406 (19) Waalwijk, C.; Dekoning, J.R.A.; Baayen, R.P.; Gams, W. Discordant groupings of
 407 *Fusarium* spp. from sections *Elegans*, *Liseola* and *Dlaminia* based on ribosomal ITS1 and ITS2
 408 sequences. *Mycologia* **1996**, *88*, 361-368.
- 409 (20) O'donnell, K.; Gueidan, C.; Sink, S.; Johnston, P.R.; Crous, P.W.; Glenn, A.; Riley, R.;
 410 Zitomer, N. C.; Colyer, P.; Waalwijk, C.; van der Lee, T.; Moretti, A.; Kang, S.; Kim, H.-S.;
 411 Geiser, D.M.; Juba, J.H.; Baayen, R.P.; Crome, M.G.; Bithel, S.; Sutton, D.A.; Skovgaard, K.;
 412 Ploetz, R.; Kistler, H.C.; Elliott, M.; Davis, M.; Sarver B.A.J. A two-locus DNA sequence database
 413 for typing plant and human pathogens within the *Fusarium oxysporum* species complex. *Fungal*
 414 *Genet. Biol.* **2009**, *46*, 936-948.

- 415 (21) Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, D.J.. Basic local alignment
416 search tool. *J. Mol. Biol.* **1990**, *215*, 403-410.
- 417 (22) NCBI BLAST; <http://www.ncbi.nlm.nih.gov/BLAST>; 22 October 2013
- 418 (23) MycoBank; <http://www.mycobank.org/BioloMICSSequences.aspx?expandparm=f&file=all>;
419 22 October 2013
- 420 (24) EMBOSS pairwise sequence alignment tool;
421 http://www.ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html; 22 October 2013
- 422 (25) Dor, E.; Evidente, A.; Amalfitano, C.; Agrelli, D.; Hershenhorn, J. ,The influence of growth
423 conditions on biomass, toxins and pathogenicity of *Fusarium oxysporum* f. sp. *orthoceras*, a
424 potential agent for broomrape biocontrol. *Weed Res.* **2007**, *47*, 345-352.
- 425 (26) Sedra, M.H.; Lazrek, B.H. , *Fusarium oxysporum* f. sp. *albedinis* toxin characterization and
426 use for selection of resistant date palm to bayoud disease. In: *Date Palm Biotechnology*, edition n.
427 XVIII; Jain, S.M., Al-Khayri, J.M., Johnson, D.V., Eds.; Springer: Netherlands, 2011; Cap 13, 253-
428 270.
- 429 (27) Megnegneau, B.; Branchard, M. Effects of fungal culture filtrates on tissue from susceptible
430 and resistant genotypes of muskmelon to *Fusarium oxysporum* f. sp. *melonis*. *Plant Sci.* **1991**, *79*,
431 105-110.
- 432 (28) Morpurgo, R.; Lopato, S.V.; Afza, R.; Novak, F.J. Selection parameters for resistance to
433 *Fusarium oxysporum* f. sp. *cubense* race 1 and race 4 on diploid banana (*Musa acuminata* Colla).
434 *Euphytica* **1994**, *75*, 121-129.
- 435 (29) Sutherland, M.L.; Pegg, G.F. Purification of a toxin from *Fusarium oxysporum* f. sp.
436 *lycopersici* race 1. *Physiol. Mol. Plant Pathol.* **1995**, *46*, 243-254.
- 437 (30) Sharma, A.; Rathour, R.; Plaha, P.; Katoch, V.; Khalsa, G.S.; Patial, V.; Singh, Y.; Pathania,
438 N. K. Induction of *Fusarium* wilt (*Fusarium oxysporum* f. sp. *pisi*) resistance in garden pea using
439 induced mutagenesis and in vitro selection techniques. *Euphytica* **2010**, *173*, 345-356.

- 440 (31) Hernandez, L.; Marangon, A.V.; Salci, T.; Svidzinski, T.I.E. , Toxic thermoresistant
441 metabolites of *Fusarium oxysporum* are capable of inducing histopathological alterations in Wistar
442 rats. *J. Venom. Anim. Toxins Incl. Trop. Dis.* **2012**, *18*, 144-149.
- 443 (32) Abraham, W.R.; Hanssen, H.P.. Fusoxysporone - a new type of diterpene from *Fusarium*
444 *oxysporum*. *Tetrahedron* **1992**, *48*, 10559-10562.
- 445 (33) Savard, M.E.; Miller, J.D.; Ciotola, M.; Watson, A.K. Secondary metabolites produced by a
446 strain of *Fusarium oxysporum* used for *Striga* control in West Africa. *Biocontrol Sci. Technol.*
447 **1997**, *7*, 61-64.
- 448 (34) Loffler, H.J.M.; Mouris, J.R. Fusaric acid - *phytotoxicity* and in vitro production by
449 *Fusarium oxysporum* f. sp. *lilii*, the causal agent of basal rot in lilies. *Neth. J. Plant Pathol.* **1992**,
450 *98*, 107-115.
- 451 (35) Gapillout, I.; Milat, M.L.; Blein, J.P.. Effects of fusaric acid on cells from tomato cultivars
452 resistant or susceptible to *Fusarium oxysporum* f. sp. *lycopersici*. *Eur. J. Plant Pathol.* **1996**, *102*,
453 127-132.
- 454 (36) Chakrabarti, D.K.; Chaudhary, K.C.B.; Correlation between virulence and fusaric acid
455 production in *Fusarium oxysporum* f. sp. *carthami*. *J. Phytopathol.* **1980**, *99*, 43-46.
- 456 (37) Barna, B.; Sarhan, A.R.T.; Kiraly, Z. The influence of nitrogen nutrition on the sensitivity of
457 tomato plants to culture filtrates of *Fusarium* and to fusaric acid. *Physiol. Plant Pathol.* **1983**, *23*,
458 257-263.
- 459 (38) Toyoda, H.; Hashimoto, H.; Utsumi, R.; Kobayashi, H.; Ouchi, S. Detoxification of fusaric
460 acid by a fusaric acid-resistant mutant of *Pseudomonas solanacearum* and its application to
461 biological-control of fusarium-wilt of tomato. *Phytopathology* **1988**, *78*, 1307-1311.
- 462 (39) Megnegneau, B.; Branchard, M. Toxicity of fusaric acid observed on callus-cultures of
463 various *Cucumis melo* genotypes. *Plant Physiol. Biochem.* **1988**, *26*, 585-588.
- 464 (40) Remotti, P.C.; Loffler, H.J.M. The involvement of fusaric acid in the bulb-rot of *Gladiolus*.
465 *J. Phytopathol.* **1996**, *144*, 405-411.

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 (Δ) 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 $\alpha =$
 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 $\alpha = 0.05$. 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-

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

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

Figure 7. Relationship between the toxins production and phytotoxicity. The graphics represent the linear correlation calculated for **1** (A, B), **2** (C, D) and the sum of both toxins (E, F) production by each *Fop* race 2 isolates with the lesion size induced by their corresponding culture filtrate (A, C, E) or their overall aggressiveness on the susceptible pea cultivar 'Messire' estimated by their AUDPC values (B, D, F). Horizontal bar are standard error for lesion size area or AUDPC values with n= 4 and n= 5 respectively while vertical bars are standard error bars of toxin content calculated with 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
J11412	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 Pflieger (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.

Figure 2

Figure 3.

A

B

C

Figure 4.**A** **B**

Figure 5.

Figure 6.

Figure 7.

SUPPORTING INFORMATION

**Identification of the Main Toxins Isolated from *Fusarium oxysporum* f. sp. *pisii* 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.

<i>Fop</i> <i>isolates</i>	Genbank number	ITS		Genbank number	EF-alpha	
		Length (bp)	Identity (%)		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
<i>Fop1</i>	KF913727	544	100	KF913732	714	99.0

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

Compound	<i>R_t</i> (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

^aCalculated in the form $y=a+bx$ where y =chromatographic peak area and x =µg of metabolite injected.

^bLimit of detection