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# UNIVERSITÀ DEGLI STUDI DI TORINO

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9 **Molecular characterization of *Fusarium oxysporum* f.sp. *cichorii* pathogenic on**  
10 **chicory (*Cichorium intybus*)**

11

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19

20 **Abstract**

21 **Keywords:** *Cichorium intybus*, wilt disease, *Fusarium oxysporum*, *formae speciales*, phylogeny.

22

23 *Fusarium oxysporum* is a ubiquitous soilborne ascomycete responsible for vascular wilt in many plant  
24 species worldwide. This species comprises more than 120 putative host-specific *formae speciales*  
25 capable of causing remarkable economical losses. In summer 2009, wilt symptoms, including chlorosis  
26 and poor development of the root system were observed on cultivars of chicory (*Cichorium intybus*) in  
27 northern Italy. The causal agent isolated from symptomatic tissues in this case was identified as *F.*  
28 *oxysporum* on the basis of both morphological features and molecular analyses. In this work, we  
29 attempted to characterize the isolates of *F. oxysporum* from *C. intybus* by both biological and  
30 molecular approaches. Pathogenicity trials performed on 5 species of the Asteraceae family with  
31 isolates of *F. oxysporum* from *C. intybus* indicated that the pathogen has a unique host range, infecting  
32 chicory only. Neither lettuce nor endive, lawn daisy and Paris daisy developed the disease. Five  
33 cultivars within *C. intybus* species were tested, and the cv Clio was the most susceptible. Phylogenetic  
34 analyses relative to the ribosomal intergenic spacer (IGS) and translation elongation factor 1-alpha  
35 (EF1- $\alpha$ ) assigned isolates pathogenic to chicory to a single cluster, distinct from other pathogenic *F.*  
36 *oxysporum*. In light of these views, we propose to designate this organism as *Fusarium oxysporum* f.sp.  
37 *cichorii*.

38

39           **Introduction**

40

41           Chicory (*Cichorium intybus* L.) is a wild perennial plant belonging to the family Asteraceae  
42 (Compositae), which has been selected, domesticated and cultivated in Europe, as well as endive (*C.*  
43 *endivia* L.), its closest related species. Within *C. intybus*, several cultivars can be distinguished, based  
44 on the use of roots or leaves (fresh or cooked) for human consumption (Bremer, 1994; Lucchin et al.,  
45 2008).

46           Chicory crops are subjected to attacks caused by several soilborne fungi, such as *Rhizoctonia*  
47 *solani* (Alfieri et al., 1984), *Phytophthora tentaculata* (Garibaldi et al., 2010), *Sclerotium rolfsii*  
48 (Alfieri et al., 1984), and *Sclerotinia sclerotiorum* (Gerlagh et al., 1998). *S. sclerotiorum*, in particular,  
49 is the major pathogen of chicory, due to its broad spectrum of virulence on several cultivars, provoking  
50 remarkable yield losses up to 32% (Benigni and Bompeix, 2010). In summer 2009, a wilt caused by *F.*  
51 *oxysporum* was observed for the first time in the world on 25-30% of 1 hectare of *C. intybus* cv. Clio  
52 plants in northern Italy. Symptoms observed included chlorosis and a poor development of the root  
53 system. The causal agent isolated from symptomatic vascular tissues was recently identified as *F.*  
54 *oxysporum* on the basis of morphological features and molecular analyses by direct sequencing of the  
55 internal transcribed spacer (ITS) rDNA region and of the translation elongation factor-1 $\alpha$  (EF-1 $\alpha$ )  
56 (Garibaldi et al., 2011).

57           *F. oxysporum* is a ubiquitous soilborne ascomycete which causes devastating vascular wilts in  
58 many plant species worldwide (Armstrong and Armstrong, 1981). The infection process starts when  
59 infection hyphae directly penetrate the specific host roots. Mycelium spreads inter- and intra-cellularly  
60 through the root cortex until it reaches the vascular vessels. As the infection spreads, the plant vessels  
61 get plugged, also due to production of vascular gels and gums as a plant defense mechanism. As a  
62 consequence, water supply is severely disrupted, and wilting, chlorosis, necrosis and eventually death  
63 occur. More than 120 different but morphologically undistinguishable *formae speciales* have been  
64 identified, based on host specificity (Katan, 1999; Michielse and Rep, 2009). In addition, *formae*  
65 *speciales* are subdivided into physiological races depending on their capability to cause wilt disease on  
66 different host cultivars (Correll et al, 1991). *Formae speciales* of *F. oxysporum* can be differentiated  
67 using pathogenicity tests with the appropriate host; however, these tests are time consuming and  
68 affected by environmental and growth culture changes (Mbofung et al., 2007). Therefore these methods

69 are often integrated or replaced by different molecular techniques which are rapidly increasing  
70 (Lievens et al., 2008), even though the molecular discrimination of *F. oxysporum* isolates is  
71 complicated by the polyphyletic nature of many *formae speciales*: some isolates of different *formae*  
72 *speciales* may be more related than isolates belonging to the same *forma specialis* (Kistler, 1997).  
73 Restriction fragment analysis of PCR-amplified ribosomal intergenic spacers (PCR-RFLP; Botstein et  
74 al., 1980) and amplified fragment length polymorphism (AFLP; Vos et al., 1995) can help to  
75 characterize large *F. oxysporum* populations (Edel et al., 1995), although the former is laborious and  
76 the latter is relatively costly. Random amplified polymorphic DNA (RAPD) analysis has also been  
77 employed (Williams et al., 1990); although the poor reproducibility of results between different  
78 laboratories is considered the major disadvantage of this technique, RAPD approach remains a  
79 relatively fast and cheap method capable of revealing major genetic variation (Bardakci, 2001) In  
80 addition, conserved housekeeping genes, such as the transcription elongation factor 1 $\alpha$  (EF-1 $\alpha$  ;  
81 Amatulli et al., 2010) and the intergenic spacer of the nuclear rRNA gene (IGS; Mbofung et al., 2007)  
82 seem to have a phylogenetic meaning (O'Donnell et al., 2009). The IGS region, which separates rDNA  
83 repeat units, is particularly suitable for studying intraspecific relationships (Mbofung et al., 2007;  
84 Srinivasan et al., 2010).

85 No study about the genetic diversity of *F. oxysporum* isolates from *C. intybus* has been  
86 reported to date. Hence, the aim of our work was to characterize the isolates of *F. oxysporum* from *C.*  
87 *intybus* by both biological and molecular approaches, correlating DNA sequence typing with the  
88 pathogenicity.

89

## 90 **Materials and methods**

91 ***F. oxysporum* isolates.** Table 1 shows details of the isolates, including codes, year of  
92 isolation, geographical origin, host source and GenBank accession numbers for IGS and TEF. Isolates  
93 from *Cichorium intybus* were maintained on slants of potato dextrose agar (PDA, Merck, Darmstadt,  
94 Germany) at 4°C.

95 **Inoculum production and pathogenicity tests.** All *F. oxysporum* isolates from infected  
96 chicory plants were grown in 100 mL of casein hydrolyzate at 25°C with 12 h of fluorescent light and  
97 shaking at 0.1 x g. After 10 days, the cultures were filtered to collect conidia which were adjusted to a  
98 final concentration 10<sup>6</sup> CFU (colony forming units)/mL with sterile deionized water.

99 All single conidium cultures identified as *F. oxysporum* were tested for pathogenicity (Table 2). Cross-  
100 inoculations on economically important hosts belonging to Asteraceae were performed in the  
101 greenhouse at 26-30°C. Different cultivars of lettuce (*Lactuca sativa*: cvs, Batavia verde), lawn daisy  
102 (*Bellis perennis*, cv. Rubyrosa), Paris daisy (*Argyranthemum frutescens*, cv Yellow), endive  
103 (*Cichorium endivia*: cvs Riccia Cuor d'oro, Scarola Verde Cuor Pieno), chicory (*C. intybus*: cvs Katia,  
104 Clio, Catalogna frastagliata, Selvatica da campo, Puntarelle, Pan di zucchero, Gigante di Chioggia)  
105 obtained from different seed companies in Italy were tested. Seeds were sown in a steamed mixture  
106 (peat: compost broad bark: sand, 60: 20: 20 vol/vol) in plug trays and maintained at 25°C. Roots of 20-  
107 day-old plants were trimmed to a length of 5 cm, and dipped for 10 min in the pathogen spore  
108 suspension prepared as described above. Inoculated seedlings were transplanted in trays (10 litres)  
109 containing steamed soil (30 min at 80°C). For ornamental species, 40-day old plants were used. Control  
110 plants were prepared similarly but soaked in plain deionized water. Five seedlings per pot were  
111 transplanted and each pot was considered as a replicate. Four replicates were used in each trial. Plants  
112 were maintained in a glasshouse at temperatures ranging between 22°C and 34°C. Symptoms started to  
113 be visible 10-12 days after artificial inoculation. Plants were evaluated weekly for disease development  
114 and wilted plants were counted. The disease index used throughout the experiments ranged from 0 to  
115 100 (0= healthy plant; 25 = initial symptoms of leaf chlorosis; 50 = severe leaf chlorosis and initial  
116 symptoms of wilting during the hottest hours of the day; 75 = severe wilting symptoms and initial  
117 symptoms of leaf chlorosis; 100 = plant totally wilted, leaves completely necrotic). Symptomatic plants  
118 showed brown or black streaks in the vascular system. The final disease rating took place five weeks  
119 after inoculation (Table 2). Data were statistically analyzed by using analysis of variance (ANOVA)  
120 and Duncan test (P<0.05). The pathogenicity test was performed at least twice.

121

122 **DNA extraction.** Genomic DNA of all studied *F. oxysporum* isolates from chicory and four  
123 different *formae speciales* (FR 30/03, ATCC 16600, ATCC 16601, ATCC 16603) was extracted from  
124 about 100 mg of mycelium scraped from PDA Petri dishes using the NucleoSpin kit (Macherey Nagel  
125 GmbH, Duren, DE, USA), according to the manufacturer's instructions, adding 10 µl of a solution of  
126 Proteinase K (10 mg mL<sup>-1</sup>) and 10 µl of RNase A (12 mg mL<sup>-1</sup>) to the lysis buffer in each tube. DNA  
127 concentration was measured with a Nanodrop 2000 (Thermoscientific, Whaltam, MA, USA) and  
128 purified DNAs were stored at -20°C.

129

130 **Random amplified polymorphic DNA (RAPD) conditions**

131 RAPD-PCR was performed using sixteen 10-mer primers, randomly chosen among OPB, OPE  
132 OPA and OPL (Operon Technologies Inc., Alameda, CA, USA). Amplification was carried out in 20  
133  $\mu\text{L}$  of a reaction mix containing 10 mM Tris-HCl, pH 9.0, 1.5 mM  $\text{MgCl}_2$ , 50 mM KCl, 0.1% triton X-  
134 100, 0.01% (wt/vol) gelatin, 60  $\mu\text{M}$  each dATP, dCTP, dGTP, and dTTP, 5 pM primer, 0.2 ng of  
135 genomic DNA, and 0.75 U of *Taq* DNA polymerase (Qiagen, Chatsworth, CA, USA). A T-Gradient  
136 thermal cycler (Biometra, Gottingen, Germany) was programmed to one cycle of 2.5 min at 94°C, 45  
137 cycles of 30 s at 94°C, 1 min at 36°C, 2 min at 72°C, with no ramping, followed by one cycle of 5 min  
138 at 72°C. RAPD analysis was repeated at least twice for each isolate. The whole reaction product (20  $\mu\text{l}$ )  
139 was loaded on a 1.5% agarose gel containing 2  $\mu\text{L}$  100  $\text{mL}^{-1}$  SYBR safe DNA gel stain (Invitrogen,  
140 Eugene, OR, USA). Moreover, the DNA of two species of *Fusarium* (*F. fujikuroi* and *F.*  
141 *verticillioides*) was included as positive control of RAPD amplification. Bench top 100 bp DNA  
142 Ladder with size ranging from 100 to 1500 base pairs was used in RAPD fingerprinting.  
143 Electrophoresis conditions were the following: 2 h 30 min at 3.3  $\text{V cm}^{-1}$  in 1 $\times$  TAE (40 mM Tris, 40  
144 mM acetate, 2 mM EDTA, pH 8.0) running buffer. Amplimers were visualized under UV light and  
145 images were acquired with a Gel Doc 1000 System (Bio-Rad, Hercules, CA, USA).

146

147 **IGS and EF-1 $\alpha$  amplification and sequencing.** The IGS sequences were amplified entirely  
148 using the primers CNL12 and CNS1 in a mixture containing 10 ng genomic DNA, 1  $\mu\text{M}$  each primer, 5  
149 U Fast Start *Taq* DNA Polymerase (Roche, Basel, Switzerland), 10  $\mu\text{L}$  colourless 10x buffer containing  
150 dNTPs and 1X GC-rich solution (Roche). A T-Gradient thermal cycler was programmed to 95°C for 5  
151 min; 38 cycles: 94°C for 1 min, 60°C for 90 sec, 72°C for 3 min; 72°C for 10 min. PCR products were  
152 electrophoresed on 0.8% agarose gel (Agarose D-1 LOW EEO Eppendorf, Hamburg, Germany) with  
153 2  $\mu\text{L}$  100  $\text{mL}^{-1}$  SYBR safe DNA gel stain (Invitrogen). PCR products were purified using a QIAquick  
154 PCR purification kit (Qiagen) and sequenced with additional internal primers (Mbofung *et al.*, 2007).  
155 For amplification of EF-1 $\alpha$ , the primer pair EF-1/EF-2 was employed; the reaction consisted of 10 ng  
156 genomic DNA, 1  $\mu\text{M}$  each primer, 1 U *Taq* DNA Polymerase (Qiagen) 10x buffer containing  $\text{MgCl}_2$   
157 and 200  $\mu\text{M}$  each dNTP. PCR program was the following: 95°C for 5 min; 35 cycles: 94°C for 45 sec,  
158 60°C for 45 sec, 72°C for 1min; 72°C for 10 min. Purified products were directly sequenced (LMU



159 sequencing service, Martinsried, Germany). The IGS and EF-1 $\alpha$  sequences obtained were deposited in  
160 GenBank (accession numbers are reported in Table 1). The primers used in this work are listed in table  
161 3.

162 **Phylogenetic analysis.** The complete IGS sequence was re-constructed using DNA Baser  
163 software v2.71.0 (Heracle Software, Lilienthal, Germany). Complete IGS and EF-1 $\alpha$  sequences were  
164 aligned using the ClustalW v.1.6 program (Kumar et al., 2008) included in the MEGA 5 software.  
165 Sequences were analyzed to determine the relationships between isolates by neighbour-joining method  
166 (Saitou and Mei, 1987). Bootstrap analysis was based on 1,000 re-samplings. A unique consensus  
167 phylogenetic tree was obtained using the SpiltsTree4 software version 4.11.3 (Huson and Bryant,  
168 2006). Fourteen IGS and ten EF-1 $\alpha$  sequences of other *formae speciales* of *F. oxysporum* were  
169 retrieved from GenBank database and used as reference sequences (marked with an asterisk in Table  
170 1).

171

## 172 **Results**

### 173 **Pathogenicity trials**

174 Results of the pathogenicity trials carried out on 5 species (12 cultivars) of the Asteraceae family with  
175 11 isolates of *F. oxysporum* from *C. intybus* are presented in Table 2. Typical symptoms of wilt disease  
176 on chicory were first observed 10-15 days after inoculation. Symptoms, including chlorosis and a poor  
177 development of the root system, were similar to those described by Garibaldi *et al.* (2011). None of the  
178 11 isolates was pathogenic on Paris daisy, lawn daisy, lettuce (two cultivars) and endive (two  
179 cultivars). All the isolates showed some level of pathogenicity on the 5 cultivars of *C. intybus* tested.  
180 However, the cv Clio was the most susceptible to the 11 isolates with a disease index ranging from 40  
181 to 65, followed by “Cicoria Katia” and “Puntarelle”. Even though the cvs Catalogna frastagliata and  
182 Selvatica da Campo showed some disease symptoms, their disease index was never statistically  
183 different from the other species tested. Among the 11 isolates, FUSCIC 45A and FUSCIC 45B were  
184 obtained in 2009 from *C. intybus* cv. Clio, while the other 9 were obtained in 2010 from *C. intybus* cv.  
185 Katia. In any case, no significant differences could be observed on the 5 cultivars tested. *F. oxysporum*  
186 was consistently re-isolated from inoculated symptomatic plants at the end of the experiments. The re-  
187 isolation was performed by using the semi-selective medium for *Fusaria* (Komada, 1975) from both  
188 symptomless and affected plants. Similar results were observed in all the trials performed.

189

190 **RAPD analysis**

191 RAPDs generated by single-primer PCR were used to compare the relationship between the isolates  
192 FUSCIC1-9, FUSCIC45A, FUSCIC45B, and *F. fujikuroi* and *F. verticillioides* as outgroups. A  
193 negative control without DNA template was included. Figure 1 shows only three gels obtained from  
194 amplifications with three representative primers (OPE-07, OPE-15 and OPL-07), out of the 16 used.  
195 Isolates from *C. intybus* presented the same pattern with each primer tested, except with a primer. In  
196 effect, a slight difference was observed for FUSCIC45A with the primer OPE-07 (red squared). A red  
197 arrow in the three Figures 1A, 1B and 1C, indicates a fragment typical of all the isolates of *F.*  
198 *oxysporum* f. sp. *cichorii*. Each analysis was repeated at least twice, and the same pattern was observed.  
199 These results led us to conclude that isolates FUSCIC1 to 9 are identical, based on the RAPD analysis  
200 carried out. Therefore no phylogenetic tree based on amplicon presence/absence was considered and  
201 for further analysis only five of these isolates were considered.

202

203 **IGS AND EF1- $\alpha$  sequencing and phylogenetic analysis.** The PCR reactions performed with IGS  
204 primers resulted in an amplicon ranging from 2,000 to 2,500 bp in the isolates tested. The internal  
205 primers generated smaller fragments that were assembled with DNA baser in order to obtained IGS  
206 contigs. The IGS sequence of each isolate was analyzed to infer the phylogenetic relationship using the  
207 Neighbour-Joining method. As for transcription elongation factor, the amplicon obtained was between  
208 650 and 700 bp and the same phylogenetic analysis was applied. Subsequently a consensus supertree  
209 was built (Figure 2).

210 Three different clusters (Groups I, II and III) were identified. The analysis supported the three groups  
211 proposed by Neighbor Joining. While Group I comprised isolates of *formae speciales lactucae* (race 2),  
212 ATCC16603-*matthioli*, NRRL22545-*matthioli*, BMP1300, BMP1301 and BMP1333 (*lactucae* race 1)  
213 clustered in Group II. Five isolates from *C. intybus* fell into group III, significantly separated from the  
214 others. Interestingly, FUSCIC45A and FUSCIC45B which were isolated in 2009 were separated from  
215 FUSCIC1-5, isolated in 2010.

216

217 **Discussion**

218           Although five members of the Asteraceae family were inoculated with the 11 isolates in  
219 analysis, different levels of susceptibility were scored only on cultivars of *C. intybus*. Therefore, we  
220 propose that the organism responsible for Fusarium wilt in *C. intybus* first observed in 2010 (Garibaldi  
221 et al., 2010) could be designated as *F. oxysporum* f.sp. *cichorii*. As described above, the fungus shows  
222 a unique host range, since vascular wilt developed severely only in *C. intybus* cv Clio and cv Katia, but  
223 not in lettuce, lawn daisy, paris daisy or endive. This conclusion based on biological tests, is supported  
224 by molecular evidences. Although bioassays are very effective, they are time-consuming and laborious.  
225 Attempts are made to replace them with molecular identification techniques to group isolates (Lievens  
226 et al., 2008; Pasquali et al., 2008). To better understand the genetic relationship between the *formae*  
227 *speciales* of *F. oxysporum* that have been already characterized and our isolates, it was useful to  
228 sequence the EF-1 $\alpha$  and IGS regions (Srinivasan et al., 2012). Sequence analysis is very precise and  
229 reproducible to evaluate genetic similarity or identity, and can separate *formae speciales* of *F.*  
230 *oxysporum* better than RAPD, which was used in this work only to assess the similarity of the eleven  
231 FUSCIC.

232 Because of the high polymorphism rate present in the IGS as well as in the EF 1- $\alpha$  sequences (Fujinaga  
233 et al., 2005; Mbofung et al., 2007), these loci allowed us to determine the diversity among *formae*  
234 *speciales* with a different host range. Our analysis indicates that the FUSCIC were phylogenetically  
235 distinct from the other isolates, although an interesting closeness with race 1 of *F. oxysporum* f.sp.  
236 *lactucae* could be detected. Bearing in mind that *F. oxysporum* f.sp *lactucae* infects lettuce, which is a  
237 member of the Asteraceae, that *C. intybus* also belongs to this family, and that the genome of *F.*  
238 *oxysporum* is highly versatile (Ma et al., 2010), the possibility that the new *forma specialis* arose from  
239 multiple recombination events taking place in known *formae speciales* including *lactucae* could be  
240 considered. A similar but mirror event has been recently reported, when it was described how *F.*  
241 *oxysporum* f.sp. *raphani* transferred its infection capability from Brassicaceae to lamb's lettuce  
242 (*Valerianella oleria*, Valerianaceae; Srinivasan et al., 2010). In the latter case the plants shared the  
243 growing environment and one *forma specialis* acquired the ability to enlarge its host range. Another  
244 example is represented by the non pathogenic strains of *Fusarium* which can be easily converted to  
245 pathogenic ones by transferring lineage-specific genes (van der Does et al., 2008) or even  
246 chromosomes. Finally, the different susceptibility of the five cvs of *C. intybus* evaluated lead to the  
247 conclusion that different physiological races might already exist.

248 Recently, the need and utility of using a multi-locus approach has been demonstrated. The  
249 disagreement between the IGS rDNA and data (O'Donnell *et al.*, 2009), provides a clear evidence  
250 against single-locus data for phylogenetic reconstruction and for inferring species limits within the  
251 Fungi (Taylor *et al.*, 2000). Although IGS rDNA has become one of the most popular loci for  
252 investigating genetic diversity within the *F. oxysporum* species complex (FOSC) and its high levels of  
253 nucleotide diversity provide a high degree of discriminatory power useful for isolate identification,  
254 sometimes the evolutionary history of this locus could obscure an accurate phylogenetic relationship  
255 within this species. The same is extended to the transcription factor EF. Other loci to be considered are  
256 for example genes for endo- and exo-polygalacturonases, mating factors and mitochondrial small  
257 subunit rDNA (mtSSU; Hirano and Arie, 2009; Wunchs *et al.*, 2009; O'Donnell *et al.*, 2011). In this  
258 work we evaluated IGS and EF-1 $\alpha$  since many of the sequences used for our analysis were already  
259 available in GenBank.

260

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264

265 **References**

- 266 Alfieri, S.A., Langdon, K.R., Wehlburg, C., & Kimbrough, J.W. (1984). Index of Plant Diseases in  
267 Florida. *Florida Department of Agriculture and Consumer Service, Division of the Plant Industry*  
268 *Bulletin*, 11, 1-389.
- 269 Armstrong, G.M., & Armstrong, J.K. (1981). *Formae speciales* and races of *Fusarium oxysporum*  
270 causing wilt diseases. In: P.E. Nelson, T.A. Toussoun, R.J. Cook R.J. (Eds.), *Fusarium: disease,*  
271 *biology and taxonomy*. University Park: State University Press, 391-399.
- 272 Amatulli, M.T., Spadaro, D., Gullino, M.L., & Garibaldi, A. (2010). Molecular identification of  
273 *Fusarium* spp. associated with bakanae disease of rice and assessment of their virulence. *Plant*  
274 *Pathology*, 59, 839-844.
- 275 Bardakci, F. (2001). Random amplified polymorphic dna (RAPD) markers. *Turkish Journal of*  
276 *Biology*, 25, 185-196.
- 277 Benigni, M., & Bompeix, G. (2010). Chemical and biological control of *Sclerotinia sclerotiorum* in  
278 witloof chicory culture. *Pest Management Science*, 66, 1332-1336.
- 279 Bremer, K. (1994). *Asteraceae: Cladistics and classification*. Portland: Timber Press.
- 280 Correll, J.C., Gordon, T.R., McCain, A.H., Fox, J.W., Koheler, C.S., et al. (1991). Pitch canker  
281 disease in California, USA: Pathogenicity, distribution and canker development on Monterey Pine,  
282 *Pinus radiata*. *Plant Disease*, 75, 676-682.
- 283 Fujinaga, M., Ogiso, H., Shinohara, H., Tsushima, S., Nishimura, N., et al. (2005). Phylogenetic  
284 relationships between the lettuce root rot pathogen *Fusarium oxysporum* f. sp. *lactucae* races 1, 2 and 3  
285 based on the sequence of the intergenic spacer region of its ribosomal DNA. *Journal of General Plant*  
286 *Pathology*, 71, 402-407.
- 287 Garibaldi, A., Gilardi, G., & Gullino, M.L. (2010). First report of collar and root rot caused by  
288 *Phytophthora tentaculata* on witloof chicory (*Cichorium intybus*) in Italy. *Plant Disease*, 94, 1504.
- 289 Garibaldi, A., Gilardi, G., Poli, A., & Gullino, M.L. (2011). First report of *Fusarium* wilt of chicory  
290 (*Cichorium intybus*) caused by *Fusarium oxysporum* in Italy. *Plant Disease*, 95, 496.
- 291 Gerlagh, M., Goossen-van de Geijn, H.M., Fokkema, N. J., & Vereijken, P.F.G. (1999). Long-  
292 Term Biosanitation by Application of *Coniothyrium minitans* on *Sclerotinia sclerotiorum*-Infected  
293 Crops. *Phytopathology*, 89, 141-147.
- 294 Hirano, Y., & Arie, T. (2009). Variation of phylogeny of *Fusarium oxysporum* isolates based on  
295 nucleotide sequence of polygalacturonase genes. *Microbes and Environments*, 24, 113-120.
- 296 Huson, D.H., & Bryant, D. (2006). Application of Phylogenetic Networks in Evolutionary Studies.  
297 *Molecular Biology and Evolution*, 23, 254-267.
- 298 Katan, T. (1999). Current status of vegetability groups in *Fusarium oxysporum*. *Phytoparasitica*,  
299 27, 51-64.
- 300 Kistler, H.C. (1997). Genetic Diversity in the Plant-Pathogenic Fungus *Fusarium oxysporum*.  
301 *Phytopatology*, 87, 474-479.
- 302 Kumar, S, Nei, M., Dudley, J., & Tamura, K. (2008). MEGA: A biologist-centric software for  
303 evolutionary analysis of DNA and protein sequences. *Briefings in Bioinformatics*, 9, 299-306.

304 Lievens B., Rep M., & Thomma, B.P.H.J. (2008). Recent developments in the molecular  
305 discrimination of *formae speciales* of *Fusarium oxysporum*. *Pest Management Science*, 64: 781-788.

306 Lucchin, M., Varotto, S., Barcaccia, G., & Parrini, P. (2008). Chicory and endive. In: J. Prohen, F.  
307 Nuez (Eds.). *Vegetables I*. (pp. 3–48). New-York: Springer.

308 Ma, L.J., van der Does, H.C., Borkovich, K.A., Coleman, J.J., Daboussi, M.J., Pietro, A.D. et al.  
309 (2010). Comparative genomics reveals mobile pathogenicity chromosomes in *Fusarium*. *Nature*, 464,  
310 367-373.

311 Mbofung, G.Y., Hong, S.G., & Pryor, B.M. (2007). Phylogeny of *Fusarium oxysporum* f. sp.  
312 *lactucae* inferred from mitochondrial small subunit, elongation factor 1- $\alpha$  and nuclear ribosomal  
313 intergenic spacer sequence data. *Phytopathology*, 97, 87-98.

314 O'Donnell, K., Kistler, H., Cigelnik, E., & Ploetz, R.C. (1998). Multiple evolutionary origins of the  
315 fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene  
316 genealogies. *Proceedings of the National Academy of Science*, 95, 2044-2049.

317 O'Donnell, K., Gueidan, C., Sink, S., Johnston, P.R., Crous, P.W., et al. (2009). A two-locus DNA  
318 sequence database for typing plant and human pathogens within *Fusarium oxysporum* species complex.  
319 *Fungal Genetics and Biology*, 46, 936-948.

320 O'Donnell, K., Humber, R.A., Geiser, D.M., Kang, S., Park, et al. (2011). Multiple evolutionary  
321 origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and  
322 mitochondrial gene genealogies. *Mycologia*, *Epub ahead of print*.

323 Pasquali, M., Saravanakumar, D., Gullino, M.L., & Garibaldi, A. (2008). Sequence-specific  
324 amplified polymorphism (SSAP) technique to analyse *Fusarium oxysporum* f. sp. *lactucae* VCG 0300  
325 isolate from lettuce. *Journal of Plant Pathology*, 90, 527-535.

326 Saitou, N., & Nei, M. (1987). The neighbour-joining method: a new method for reconstructing  
327 phylogenetic trees. *Molecular Biology and Evolution*, 4, 406-425.

328 Srinivasan, K., Gilardi, G., Spadaro, D., Garibaldi, A., & Gullino M.L. (2010). Molecular  
329 characterization through IGS sequencing of *formae speciales* of *Fusarium oxysporum* pathogenic on  
330 lamb's lettuce. *Phytopathologia Mediterranea*, 49, 309-320.

331 Srinivasan, K., Spadaro, D., Gilardi, G., Gullino, M.L., & Garibaldi, A. (2012). Genetic diversity  
332 and pathogenicity of *Fusarium oxysporum* isolated from wilted rocket plants in Italy. *Phytoparasitica*,  
333 *in press*.

334 Taylor, J.W., Jacobson, D.J., Kroken, S., Kasuga, T., Geiser, D.M., et al. (2000). Phylogenetic  
335 species recognition and species concepts in fungi. *Fungal Genetics and Biology*, 1, 21-32.

336 van der Does, H.C., Lievens, L., Claes, L., Houterman, P.M., Cornelissen, B.J.C., & Rep, M.  
337 (2008). The presence of a virulence locus discriminates *Fusarium oxysporum* isolates causing tomato  
338 wilt from other isolates. *Environmental Microbiology*, 10, 1475–1485.

339

## 340 TABLES

341

342 **TABLE 1.** List of strains used in this work. Codes, *formae speciales*, origin of isolation, specific host,343 accession numbers relative to IGS and EF-1 $\alpha$  sequences. n.d. = not determined.

<b>Code</b>	<b><i>F.oxysporum</i> f.sp.</b>	<b>Geographic origin</b>	<b>Year of isolation</b>	<b>Specific host</b>	<b>IGS GenBank accession number</b>	<b>TEF GenBank accession number</b>
<b>FUSCIC1</b>	<i>Unknown</i>	Italy	2010	<i>Cichorium intybus</i> L cv Katia	JQ278594	JQ278587
<b>FUSCIC2</b>	<i>Unknown</i>	Italy	2010	<i>Cichorium intybus</i> L cv Katia	JQ278595	JQ278588
<b>FUSCIC3</b>	<i>Unknown</i>	Italy	2010	<i>Cichorium intybus</i> L cv Katia	JQ278596	JQ278589
<b>FUSCIC4</b>	<i>Unknown</i>	Italy	2010	<i>Cichorium intybus</i> L cv Katia	JQ278597	JQ278590
<b>FUSCIC5</b>	<i>Unknown</i>	Italy	2010	<i>Cichorium intybus</i> L cv Katia	JQ278598	JQ278591
<b>FUSCIC6</b>	<i>Unknown</i>	Italy	2010	<i>Cichorium intybus</i> L cv Katia	-	-
<b>FUSCIC7</b>	<i>Unknown</i>	Italy	2010	<i>Cichorium intybus</i> L cv Katia	-	-
<b>FUSCIC8</b>	<i>Unknown</i>	Italy	2010	<i>Cichorium intybus</i> L cv Katia	-	-
<b>FUSCIC9</b>	<i>Unknown</i>	Italy	2010	<i>Cichorium intybus</i> L cv Katia	-	-
<b>FUSCIC45A</b>	<i>Unknown</i>	Italy	2009	<i>Cichorium intybus</i> L cv Clío	JQ278599	JQ278592
<b>FUSCIC45B</b>	<i>Unknown</i>	Italy	2009	<i>Cichorium intybus</i> L cv Clío	JQ278600	JQ278593
<b>FOA50</b>	<i>asparagi</i>	Australia	n.d	<i>Asparagus officinalis</i>	DQ831886*	DQ837691*
<b>NRRL22536</b>	<i>callistephi</i>	Germany	n.d	<i>Callistephus chinensis</i>	DQ831897*	DQ837679*
<b>ATCC16600</b>	<i>conglutinans</i>	South Carolina	n.d	<i>Brassica oleracea</i>	GQ914766*	JQ315229
<b>BMP1300</b>	<i>lactucae</i> (race1)	Wellton, Az	n.d	<i>Lactuca sativa</i>	DQ831864*	DQ837658*
<b>BMP1301</b>	<i>lactucae</i> (race1)	Wellton, Az	n.d	<i>Lactuca sativa</i>	DQ831865*	DQ837659*
<b>BMP1333</b>	<i>lactucae</i> (race1)	Wellton, Az	n.d	<i>Lactuca sativa</i>	DQ831873*	DQ837667*
<b>F9501</b>	<i>lactucae</i> (race2)	Japan	n.d	<i>Lactuca sativa</i>	DQ831863*	DQ837657*
<b>FK09701</b>	<i>lactucae</i> (race2)	Japan	n.d	<i>Lactuca sativa</i>	DQ831893*	DQ837693*
<b>FOLR2</b>	<i>lycopersici</i>	California	n.d	<i>Solanum lycopersicum</i>	DQ831894*	DQ837692*
<b>ATCC16603</b>	<i>matthioli</i>	USA	n.d	<i>Matthiola incana</i>	GQ914769*	JQ315231
<b>ATCC16603</b>						
<b>NRRL22546</b>	<i>medicaginis</i>	SE Asia	n.d	<i>Medicago sativa</i>	DQ831901*	DQ837690*
<b>ATCC16601</b>	<i>raphani</i>	USA	n.d	<i>Rhapanus sativus</i>	GQ914765*	JQ315230
<b>FR-30/3</b>	<i>raphani</i>	Italy	2003	<i>Rhapanus sativus</i>	GU001853*	JQ315232
<b>NRRL26871</b>	<i>spinaciae</i>	Japan	n.d	<i>Spinacia oleracea</i>	DQ831888*	DQ837687*

344

345 **TABLE 2.** Results of pathogenicity test. Disease index (DI) for the isolates listed. Disease index was assessed on a 0 to 100 scale. 0: healthy plants; 12.5: plants growing  
 346 regularly with slight vascular discoloration; 25: slight leaf chlorosis and reduced growth, vascular discoloration; 50: chlorosis, 50% growth reduction respect the  
 347 healthy control, vascular discoloration, initial symptoms of wilting; 75: extended vascular discoloration, strong leaf chlorosis, severe growth reduction and wilting  
 348 symptoms; 100: plants totally wilted and then dead. Analysis of variance (ANOVA) was performed and Tukey test ( $P<0.05$ ).

Disease index (0-100%)												
Species	Cv	FC1	FC2	FC3	FC4	FC5	FC6	FC7	FC8	FC9	FC45A	FC45B
<i>A. frutescens</i>	Yellow	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
<i>B. perennis</i>	Rubyrosa	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
<i>C. endivia</i>	Riccia Cuor D'oro	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
<i>C. endivia</i>	Scarola Verde Cuor Pieno	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
<i>C. intybus</i>	Catalogna Frastagliata	16 ab	25 ab	18 ab	20 ab	20 ab	15 ab	26 ab	24 ab	19 ac	30 ac	31 ab
<i>C. intybus</i>	Selvatica da campo	20 ab	21 ab	5 ab	19 ab	22 ab	21 ab	20 ab	15 ab	15 ab	15 ab	20 ab
<i>C. intybus</i>	Puntarelle	39 b	34 ab	26 ab	46 b	40 b	39 ab	17 ab	37 ac	30 bc	39 bc	33 ab
<i>C. intybus</i>	Clio	50 b	49 b	65 c	54 b	53 b	45 b	40 b	60 c	63 c	65 c	57 b
<i>C. intybus</i>	Katia	41 b	41 ab	40 bc	41 b	38 ab	25 ab	30 ab	45 bc	26 bc	45 bc	42 b
<i>C. intybus</i>	Pan di zucchero	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
<i>Cichorium intybus</i>	Cicoria Gigante Chioggia	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
<i>L. sativa</i>	Batavia Verde	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a

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350





352 **TABLE 3.** List of primers used in this study. The sequence of only three RAPD primers is reported,  
 353 the others, randomly chosen among OPB, OPE OPA and OPL (OPA02, OPA03, OPA05,  
 354 OPA15, OPA17, OPA18, OPA19, OPB01, OPB03, OPB04, OPB07, OPB13 and  
 355 OPE02 can be retrieved from Operon Technologies, (Alameda, CA, USA).  
 356

Primer	Sequence (5'→3')	Target gene	Reference
CNL12	CTGAACGCCTCTAAGTCAG	IGS fragment	Anderson and Stasovski, 1992
CNS1	GAGACAAGCATATGACTACTG	IGS fragment	White et al., 1990
U46.67	AATACAAGCACGCCGACAC	IGS fragment	Mbofung et al., 2007
RU46.67	GTGTCGGCGTGCTTGTATT	IGS fragment	Mbofung et al., 2007
CN61	GGTTCAATTTGATGTCGGCT	IGS fragment	Mbofung et al., 2007
RCN61	AGCCGACATCAAATTGACC	IGS fragment	Mbofung et al., 2007
RU3	GTGTGAAATTGGAAAGTCGG	IGS fragment	Mbofung et al., 2007
RRU3	CCGACTTTCCAATTTACAC	IGS fragment	Mbofung et al., 2007
CN34	CCAACACATGGGTGGTACCG	IGS fragment	Mbofung et al., 2007
IGSF4	CCAGACTTCCACTGCGTGTC	IGS fragment	Mbofung et al., 2007
CNS12	GCACGCCAGGACTGCCTCGT	IGS fragment	Mbofung et al., 2007
EF-1 (forward)	ATGGGTAAGGAGGACAAGAC	Translation elongation factor 1 $\alpha$	O'Donnel et al., 1998
EF-2 (reverse)	GGAAGTACCAGTGATCATGTT	Translation elongation factor 1 $\alpha$	O'Donnel et al., 1998
OPE07	AGATGCAGCC		
OPE15	ACGCACAACC		
OPL07	AGGCGGGAAC		

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359 **FIGURE CAPTIONS**

360

361 **FIGURE 1.** Random amplified polymorphic DNA (RAPD) pattern obtained from 11 isolates of *F.*  
362 *oxysporum* extracted from *C. intybus* and two reference strains with primers OPE-07 (**A**), OPE-  
363 15 (**B**) and OPL-07 (**C**). M: molecular weight marker (100 bb DNA Ladder; Qiagen); NC: no  
364 template control. 1-9: FUSCIC1- FUSCIC9; 10-11: FUSCIC45A-FUSCIC45B; 12-13: *F.*  
365 *fujikuroi* and *F. verticillioides*, respectively. Red arrow indicates an amplicon that appears only  
366 in the strains of *F. oxysporum* f. sp. *cichorii* isolated in 2010.

367

368 **FIGURE 2.** Consensus phylogenetic tree relative to IGS and EF-1 sequences of strains listed in table 1.  
369 Individual trees (Neighbour-Joining method, bootstrap analysis (1000)) were joined in a single  
370 consensus tree through consensus Supernetwork using Splitstree4 software.