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Genetic diversity and pathogenicity of Fusarium oxysporum isolated from wilted rocket plants in Italy.

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9 **Genetic diversity and pathogenicity of *Fusarium oxysporum* isolated from wilted**

10 **rocket plants in Italy**

11

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Abstract Thirty-six isolates of *Fusarium oxysporum* originated from *Eruca vesicaria* and *Diplotaxis tenuifolia* together with eight reference strains belonging to the *formae speciales raphani, matthioli* and *conglutinans*, typical on the Brassicaceae family, were tested for pathogenicity on two species of rocket plants (*E. vesicaria* L., syn. *E. sativa*, cv. 'Rucola coltivata' and *D. tenuifolia* cv. Winter) cultivated in glasshouse. The results showed that different isolates were slightly, moderately or highly virulent. The strains were examined for differences in the nucleotide sequence of the ribosomal DNA (rDNA) intergenic spacer (IGS) region, about 2.5 kb long. The phylogenetic (neighbor-joining) analysis performed on the isolates permitted to identify four different groups, named I, II, III and IV. Thirty-one isolates out of thirty-six clustered in group I and were genetically similar to *F. oxysporum* f. sp. *raphani*. By considering the pathogenicity of the strains included in Group I, a partial host specialization could be observed: the average disease index of the isolates from *D. tenuifolia* was higher on wild rocket, while the average disease index of the isolates from *E. vesicaria* was higher on cultivated rocket. Moreover, isolates from cultivated rocket showed, on average, a higher degree of aggressiveness compared to the isolates from wild rocket. About group I, the sequence analysis confirmed the homogeneity of the population, with only 5 parsimony informative SNPs and 5 haplotypes. Twenty-six out of 31 isolates belonged to haplotype 1. Group II and III were genetically similar to strains of *F. oxysporum* f.sp. *matthioli.* Three other strains, not pathogenic or with medium level of virulence clustered together in Group 4, but their sequence was distant from that of other *formae speciales*. The pathogenicity and IGS analysis confirmed the presence of virulence variation and genetic diversity among the *F. oxysporum* isolates studied. To our knowledge, this is the first report of differentiation of *formae speciales* of *F. oxysporum* on rocket plants by IGS analysis.

Keywords *Diplotaxis* spp., *Eruca vesicaria, formae speciales,* Fusarium wilt, phylogeny, ribosomal intergenic spacer

46 **Introduction**

47

48 Rocket (or arugula) is a vegetable increasingly grown in Italy and widely used in the
49 Mediterranean cuisine as salad and/or as a garnish. For centuries the plant has been known for its
50 aphrodisiac properties, as well as for pharmaceutical uses and as a carrier of drought resistance
51 (Padulosi and Pignone, 1997). Two types of rocket are available on the Italian market: (1) *Eruca*
52 *vesicaria* L. (syn. *E. sativa*), known as "ruchetta" or cultivated garden rocket and (2) several species of
53 *Diplotaxis* (*Diplotaxis erucoides*, *D. muralis* and *D. tenuifolia*), which are wild plants now widely
54 cultivated (Santamaria *et al.,* 2002; Garibaldi *et al.,* 2003). Rocket is mainly grown in southern Europe,
55 the Middle East and South-East Asia (Padulosi and Pignone, 1997).

56 In summer 2001 and spring 2002, plants of *E. vesicaria* and *D. tenuifolia* showing wilt
57 symptoms were observed in several commercial greenhouses near Bergamo, Lombardy, northern Italy.
58 Fusarium wilt was first reported in cultivated and wild rocket in several commercial plastic
59 greenhouses in Bergamo, and the causal agent was determined as *Fusarium oxysporum* (Garibaldi *et*
60 *al.,* 2003; Catti *et al.,* 2007). A wilt of *E. sativa* attributed to *F. oxysporum* f. sp. *erucae* was earlier
61 reported in India in 1973 and 1987 (Chatterjee and Rai, 1974; Gupta, 1988). New epidemics were also
62 reported in 2003 and 2004, expanding from Lombardy to Piedmont region, northern Italy (Garibaldi *et*
63 *al.,* 2006). The sudden appearance of this disease on 40 farms in the Lombardy region and later on
64 other farms in northern Italy indicated that the pathogen was seed-transmitted (Garibaldi *et al.,* 2003,
65 2006). Symptoms, including stunting and chlorosis, with brown or black streaks in the vascular system,
66 were described by Garibaldi *et al.,* 2003. Wilted rocket plants were frequently found in a large number
67 of plastic greenhouses in the major rocket growing areas in Italy.

68 This new wilt developed in the same areas where similar diseases had been observed
69 previously: wilt of lettuce, incited by *F. oxysporum* f. sp. *lactucae* (Garibaldi *et al.,* 2002), and wilt of
70 lamb's lettuce, caused both by *F. oxysporum* f. sp. *raphani* and *F. oxysporum* f. sp. *conglutinans*
71 (Gilardi *et al.,* 2008; Srinivasan *et al.*, 2010).

72 With over 80 putatively plant host-specific *formae speciales* described (Katan, 1999),
73 members of the *Fusarium oxysporum species complex* (FOSC) collectively represent the most
74 commonly encountered and economically important species complex within the *Fusarium* genus. The
75 *formae speciales* of *F. oxysporum* can be differentiated by pathogenicity tests with appropriate host and

76 by determining the vegetative compatibility groups (VCGs) obtained from heterokaryon formation
77 between anastomosing nitrate non-utilizing (*nit*) mutants (Garibaldi *et al.,* 2006; Catti *et al.,* 2007). The
78 results suggest the presence of genetic diversity among these isolates.

79 The tests are however time consuming, labor intensive and subject to varying environmental
80 or culture growth conditions during the experiments (Woo *et al.,* 1996; Mbofung *et al.,* 2007).
81 Molecular techniques, such as restriction fragment analysis of PCR-amplified ribosomal intergenic
82 spacers (IGS), can help to rapidly characterize large *F. oxysporum* populations (Edel *et al.,* 1995). Of
83 the relatively small number of genes that have been used for phylogenetic studies within the *Fusarium*
84 genus (ex., IGS rDNA, elongation factor-1α, polygalacturonases, mitochondrial small subunit
85 ribosomal RNA, phosphate permease, β-tubulin, nitrate reductase, MAT 1 and MAT2), the EF-1α
86 (Amatulli *et al.,* 2010) and IGS rDNA genes (Mbofung *et al.,* 2007) seem adapt for such application
87 (O'Donnell *et al.*, 2009). The IGS region, which separates rDNA repeat units, is particularly suitable
88 for studying intraspecific relationships (Appel and Gordon, 1996; Mbofung *et al*., 2007). Phylogenetic
89 analysis of the IGS region sequence is very useful to study the composition of *F. oxysporum*
90 populations (Kawabe *et al.,* 2005; 2007; Enya *et al.,* 2008; Dissanayake *et al.,* 2009; Srinivasan *et al*.,
91 2010).

92 No study of *F. oxysporum* genetic diversity on isolates from cultivated and wild rocket plants
93 has been reported to date. Our main objectives were to characterize the pathogenicity of the isolates, to
94 differentiate the isolates of *F. oxysporum* from wilted rocket in Italy according to the IGS region
95 sequence variation and to correlate the sequence differences with the pathogenicity.

96

97 **Materials and methods**

98

99 ***F. oxysporum* isolates.** Table 1 shows details of the isolates, including their names, year of
100 isolation, geographical origin and original host. All isolates were maintained on slants of potato
101 dextrose agar (PDA, Merck, Darmstadt, Germany) at 4°C. The pathogenicity of some of the isolates
102 from rocket and other crops were previously determined (Garibaldi *et al.,* 2006; Catti *et al.,* 2007).

103

104 **Inoculum production and pathogenicity tests.** The *F. oxysporum* isolates from cultivated
105 and wild rocket and the reference strains of *F. oxysporum* were grown in 100 ml of casein hydrolysate

106 at 25°C with shaking at 0.1 x g. After 10 days, the cultures were aseptically filtered to collect conidia
107 which were bought to a final density of 10^6 conidia/ml in sterile water. Surface sterilized seeds of
108 cultivated rocket (cv. 'Rucola coltivata', Furia seed company) and wild rocket (cv. 'Winter', Orosem
109 seed company) were sown in a steamed substrate (peat:perlite 70:30 v/v) in plug trays (Oktpac 160,
110 Arca, Bergamo, Italy) kept at 25°C, with 12 hours of fluorescent light per day. Roots of 15-day-old
111 plants were washed, trimmed to a length of 5 cm and dipped in 200 ml of spore suspension (10^4 conidia
112 ml^{-1}) of the isolate for 10 min. Inoculated plants were then transplanted into 10-l containers filled with
113 a steamed substrate (peat:clay:perlite, 70:20:10 v/v). The control plants were prepared similarly and
114 soaked in sterile deionized water. Fifteen plants were used per isolate, arranged in a randomized block
115 design. Three replication were adopted during the study. Not inoculated plants, maintained in the same
116 glasshouse, served as healthy control. Plants, in a glasshouse at 25°C to 30°C, were watered and
117 fertilized according to local practice. All isolates were tested at least twice. Each trial lasted 32 to 42
118 days.

119 Starting 10 days after inoculation, plants were checked for symptoms at 7-day intervals and
120 wilted plants were counted. At the end of the experiments, re-isolation was carried out from inoculated
121 and control plants. A disease index was adopted to measure the plants every week; 0 corresponded to
122 healthy plants; 25 to vascular discoloration, slight leaf chlorosis and growth reduced of around 25%; 50
123 to vascular discoloration, chlorosis and strong growth reduction; 100 to dead plants. Data were
124 expressed as percent of dead plants and as disease index (0 - 100). Data from replications of repeated
125 experiments were pooled and analyzed together. Means value of disease index for each treatment were
126 reported. Data was analyzed using SPSS software (SPSS Inc., version 17.0, Chicago, IL, USA).
127 Statistical significance was judged at the level of p-value < 0.05. When the analysis of variance was
128 statistically significant, Tukey's test was used to separate the means.
129

130 **DNA extraction.** The genomic DNA of the 36 *F. oxysporum* isolates from cultivated and wild
131 rocket and the 8 reference strains was extracted from about 100 mg of mycelium scraped from Petri
132 dishes using the NucleoSpin kit (Macherey Nagel GmbH and Co., Duren, DE, USA), according to the
133 manufacturer's instructions, adding 10 µl of a solution of Proteinase K (10 mg ml^{-1}) and 10 µl of
134 RNAse A (12 mg ml^{-1}) to the lysis buffer in each tube. Purified DNAs were stored at 4°C.
135

136 **IGS sequencing.** The IGS sequences were amplified using the primers CNL12
137 (CTGAACGCCTCTAAGTCAG) and CNS1 (GAGACAAGCATATGACTACTG) in a mixture
138 containing 10 ng genomic DNA, 1 μM each primer, 5 U Fast Start Taq DNA Polymerase (Roche,
139 Basel, Switzerland) and 10 μl colorless 10x buffer containing dNTPs (Roche). A T-Gradient thermal
140 cycler (Biometra, Gottingen, Germany) was programmed to 95°C for 5 min; 38 cycles: 94°C for 1 min,
141 60°C for 90 sec, 72°C for 3 min; 72°C for 10 min. PCR products were electrophoresed on 0.8%
142 agarose gel (Agarose D-1 LOW EEO Eppendorf, Hamburg, Germany) with 1μl 100 ml^{-1} SYBR safe
143 DNA gel stain (Invitrogen, Eugene, OR, USA). Gel images were acquired with a Gel Doc EC (Bio-
144 Rad, Hercules, CA, USA).

145 PCR products were purified using a QIAquick PCR purification kit (Qiagen). They were
146 ligated with a pDrive cloning vector using the Qiagen cloning kit according to the manufacturer's
147 instructions, in 10 μl ligation mixture. After 2 h incubation, 1 - 2 μl ligation mixtures were inoculated
148 in tubes containing Qiagen EZ competent cells, incubated on ice for 5 min, heated at 42°C for 30 sec
149 without shaking, and incubated on ice for 2 min. Two hundred and fifty μl SOC (Super Optimal broth
150 with Catabolite Repression) medium (Hanahan, 1983) were added to the tubes and the contents were
151 directly plated with each transformation mixture onto Luria-Bertani medium (LB) plates containing
152 100 μg ml^{-1} ampicillin (Merck), 30 μg ml^{-1} kanamycin (Merck), 50 μg ml^{-1} IPTG, and 80 μg ml^{-1} X-
153 Gal at 37°C for 15 - 18 h for blue/white screening of recombinant colonies. The white colonies were
154 screened using M13 forward (GTTTCCCAGTCAGAC) and reverse (AACAGCTATGACCATG)
155 primers to check the presence of inserts. PCR was performed in a mixture containing 60 μM each
156 dATP, dCTP, dGTP and dTTP, 1 μM each primer, 1 Unit Taq DNA polymerase (Qiagen) and 2 μl 10x
157 colorless buffer using whole cells as template. The PCRs were run as described above but decreasing
158 the annealing temperature to 50°C.

159 About 5 ml of LB medium containing the antibiotics described for plates were inoculated with
160 a single transforming colony and incubated at 37°C for 16 h. Plasmids were purified from bacterial
161 cells following the manufacturers instruction (Qiagen) and sequenced by the BMR Genomics Centre
162 (Padova, Italy) using the ABI PRISM 3730Xl DNA Sequencer. The complete sequence of the IGS
163 region required the use of additional internal primers and sequencing was performed in both directions
164 (Mbofung *et al.,* 2007). The IGS sequences obtained were deposited in GenBank (accession numbers
165 are reported in Table 1).

166 **Phylogenetic analysis.** The IGS sequences were aligned using the program DNA Baser
167 v2.71.0 (Heracle Software, Lilienthal, Germany). Sequence alignments were manually adjusted to
168 remove mismatches. Phylogenetic analysis was conducted using MEGA version 4 software (Tamura *et*
169 *al.,* 2007). IGS sequence data were analyzed to determine the relationships by the distance and
170 parsimony methods. Nine IGS sequences of the strains of *F. oxysporum* isolated from other plant
171 species obtained from GenBank database were used as reference sequences and out-groups (Table 1).
172 A distance matrix for the aligned sequences was calculated by using the Kimura two parameter model
173 (1980) and analyzed with the neighbor-joining method (Saitou and Nei, 1987) using the program
174 ClustalW v.1.6 (Thompson *et al.,* 1994), excluding positions with gaps and missing data (complete
175 deletion option). Bootstrap analysis was based on 10,000 re-samplings.
176
177 **SNP and haplotype identification in the strains of *F. oxysprum* f.sp. *raphani.*** The
178 complete IGS sequences of the 31 *F. oxysporum* isolates belonging to Group I were aligned with the 6
179 reference strains belonging to the *forma specialis raphani*. The SNPs and haplotypes were identified
180 from contig chromatograms using the software Sequencher (Gene Codes, Ann Arbor, MI, USA). The
181 complete analysis included single-base substitutions, single-base and multi-base deletion/insertion.
182 Only parsimony informative SNPs were considered.
183
184 **Results**
185
186 **Pathogenicity test.** Results of the pathogenicity trials carried out on cultivated and wild
187 rocket with 36 isolates of *F. oxysporum* along with 8 reference strains are presented in Table 1. Typical
188 symptoms of Fusarium wilt were first observed 12 to 14 days after inoculation. Symptoms, including
189 stunting and chlorosis of plants, with brown or black streaks in the vascular system, were similar to
190 those described by Garibaldi *et al.* (2003). Out of 36 isolates, 19 showed on cultivated rocket a high
191 level of virulence (disease index between 61% and 100%), 12 a medium level of virulence (disease
192 index between 31% and 60%), 4 a low level of virulence (disease index between 10% and 30%) and
193 one isolate (FR-15A/02) resulted not pathogenic on cultivated rocket. Most of the highly virulent
194 isolates originated from *E. vesicaria*. Among the reference strains, the three *F. oxysporum* f.sp. *raphani*
195 showed a medium level of virulence, ranging from 42% to 56%. Interestingly, the three *F. oxysporum*

196 f.sp. *conglutinans* were highly virulent, with a disease index ranging from 83% to 100%. A disease
197 index of 15% and 19% was observed for the reference strains of *F. oxysporum* f. sp. *matthioli* race 1
198 ATCC16602 and race 2 ATCC16603, respectively. When the same 36 isolates were tested on wild
199 rocket, 29 isolates showed a high level of virulence, 5 showed a medium level of virulence, and two
200 (FR 15A/02 and FR 10B/02) were not pathogenic. The three reference *F. oxysporum* f.sp. *conglutinans*
201 were highly or medium virulent, with a disease index ranging from 40% to 92%. The reference strains
202 *F. oxysporum* f. sp. *matthioli* race 1 ATCC16602 and race 2 ATCC16603 were respectively not
203 pathogenic and highly pathogenic on wild rocket. Generally, the isolates were more virulent on wild
204 rocket, so that wild rocket (cv. 'Winter') could be considered more susceptible to Fusarium wilt than
205 cultivated rocket (cv. 'Rucola coltivata'). The result obtained for the two cultivars should be not
206 generalized to the other cultivars of wild or cultivated rocket and wider pathogenicity tests are needed,
207 because there is likely variation in the resistance of different cultivars within each rocket species. *F.*
208 *oxysporum* was consistently re-isolated from inoculated plants at the end of the experiments. The re-
209 isolation has been performed by using the semi-selective media for *Fusaria* (Komada, 1975) from both
210 symptomless and affected plants (Garibaldi *et al*., 2004). Similar results were observed in all the trials
211 performed.
212
213 **IGS sequencing and phylogenetic analysis.** The PCR reactions resulted in a product varying from
214 2,493 to 2,655 bp in the isolates tested. The internal primers generated smaller fragments (ranging from
215 800 to 1000 bp) with the exception of CNL12 used only to amplify the IGS sequence in each isolate.
216 The assembly of the IGS contigs and their alignment provided a sequence in the final data set of 1,472
217 bp, 134 of them being parsimony-informative. The complete IGS sequence of each isolate was
218 analyzed to infer the phylogenetic relationship using the Neighbor-Joining method (Fig. 1). Four
219 different clusters (Groups I, II, III and IV) were identified. Maximum Parsimony (MP) analysis found
220 the tree length to be 261; the consistency index was 0.912 (0.872), the retention index was 0.933
221 (0.933) and the composite index was 0.851 (0.814) for all the sites and between parentheses for the
222 parsimony-informative sites. The analysis supported the four groups proposed by Neighbor Joining.
223 Thirty-one isolates (Figure 1) collected from rocket in Italy from 2002 to 2006 as well as *F.*
224 *oxysporum* f. sp. *raphani* ATCC58110 and ATCC16601, *F. oxysporum* 6-MYA3041 and the reference
225 *raphani* strains (R238, NRRL22553 and 100017) clustered in the "*raphani*" group (Group I), with a

226 strong bootstrap value of 92%, while the remaining isolates were separated from this group by the
227 absence of the consensus sequences GTCCAAGGTA (position 550-600). They differed just by few
228 single nucleotide polymorphisms (SNPs), distributed in different positions (Table 2). The SNPs were
229 the most common differences among the pathogenic isolates. In SNP discovery, a false SNP call can
230 result from alignment errors, sequencing errors, or from defects in the SNP detection algorithm. By
231 considering only the parsimony informative SNPs (5), five haplotypes could be identified in Group I.
232 Haplotype 1 was the most common haplotype and included 26 isolates. The other 5 isolates were
233 grouped in 4 haplotypes and constituted variants of haplotype 1.

234 Interestingly, by considering the pathogenicity on wild and cultivated rocket of the strains
235 included in Group I, belonging to the *forma specialis raphani*, the average disease index of the 19
236 isolates from *D. tenuifolia* was 74.5% on *D. tenuifolia* and just 56.1% on *E. vesicaria* ($P = 0.0001$, t-
237 test). On the contrary, the average disease index of the 12 isolates from *E. vesicaria* was 90.9% on *E.*
238 *vesicaria* and just 84.0% on *D. tenuifolia* ($P = 0.047$, t-test). Isolates from cultivated rocket showed on
239 average a higher degree of aggressiveness compared to the isolates from wild rocket.

240 Other isolates from rocket did not cluster in a single group. Interestingly, the sequence of the
241 isolate FR-20/03 was very close to the strain NRRL38334 belonging to the *forma specialis matthioli*
242 and they clustered together in Group II with a bootstrap value of 99%. They were characterized by the
243 absence of the consensus sequences CTGAACGCCTCTAAGTCAGAATCCATGC (position 1 to 50
244 bp). Group III was composed by the isolate FR-4A/02 together with the reference strains ATCC16602,
245 ATCC16603 and 880120h belonging to the *forma specialis matthioli*, and they were separated from
246 Groups I and II by the presence of the consensus sequences TCCAGGGTA (position 550 to 600 bp),
247 with a bootstrap value of 88%.

248 The isolates FR-10B/02, FR-15A/02 and FR-17/03 were not similar to other isolates or
249 reference strains tested and they clustered in Group IV, with a bootstrap value of 100%, characterized
250 by the absence of the consensus sequences CCTGTCGA (position 450 to 500 bp), CGAGGGTG
251 (position 550 to 600 bp), GACGGGT and GTCGAGACGGGATGTGACGGGTG (position 650 to 700
252 bp), CGAATTGAG (position 700 to 750 bp) and also by the presence of an insertion that contains the
253 consensus sequences of CCTATACGTGCAAGATGGTTTTGCGG (position 850 to 900 bp).

254 The reference strains ATCC16600, ATCC52557 and ATCC58385 belonging to the *forma*
255 *specialis conglutinans* formed a distinct group separated from the other isolates, with a bootstrap value
256 of 58%.
257
258 **Discussion**
259
260 *Eruca vesicaria* (cultivated rocket) and *Diplotaxis* spp. (wild rocket) are two members of the
261 Brassicaceae family. Three *formae speciales* of *Fusarium oxysporum* (*raphani, conglutinans* and
262 *matthioli*) are known as being pathogenic on the species of the Brassicaceae family. After the first
263 Fusarium wilts, caused on cultivated and wild rocket by *F. oxysporum*, we created a collection of 36
264 isolates, originated from 2002 to 2006. By sequencing the ribosomal region IGS, we could effectively
265 differentiate the three *formae speciales* of *F. oxysporum* attacking the Brassicaceae and we could
266 attribute most of the highly virulent isolates originated from rocket to the *forma specialis raphani*.
267 Although bioassays are very effective, they are time-consuming and laborious. Attempts are
268 made to replace them with molecular identification techniques to group isolates (Lievens *et al.,* 2008;
269 Pasquali *et al.,* 2008). To understand the genetic relationship among the *formae speciales of F.*
270 *oxysporum*, it was useful to sequence the IGS region of several isolates of *F. oxysporum* (Srinivasan *et*
271 *al.,* 2010). IGS sequence analysis is very precise and reproducible to evaluate genetic similarity or
272 identity and can separate *formae speciales* and physiological races of *F. oxysporum* better than RAPD
273 and other molecular techniques. Recently, Fujinaga *et al.* (2005) reported that IGS sequences were a
274 useful indicator of physiological races for *F. oxysporum*. The polymorphism of this region was higher
275 than that of other loci, such as the mitochondrial Small Subunit (mtSSU) or the Elongation Factor 1-α
276 (EF) (Mbofung *et al.,* 2007), and allowed the genetic diversity detected by VCG in *Fusarium*
277 *oxysporum* f. sp. *lactucae* (Fujinaga *et al.,* 2005) to be distinguished.
278 Our phylogenetic analysis suggests that the strains of *F. oxysporum* f. sp. *raphani* were
279 phylogenetically distinct from the other isolates and are a quite homogeneous population. The results
280 indicate that the genetic diversity in the IGS sequences was extremely low within the same cluster.
281 Thirty-one pathogenic *F. oxysporum* f. sp. *raphani* isolates fell into Group I, consistently supported by
282 bootstrapping of 92%. The high level of homogeneity and the relatively low number of parsimony
283 informative SNPs and haplotypes found in the population of Group I, suggests that the *F. oxysporum*

284 isolates belonging to the *forma specialis raphani* may have a monophyletic origin and confirm their
285 recent introduction in Italy, probably with a seed lot contaminated as the pathogen is seedborne and
286 seed-transmitted. In most studies in which genetic markers have been successfully used to
287 distinguish indigenous pathogen populations from introduced ones (Engelbrecht *et al.*, 2007),
288 low levels of genetic variation have been attributed to a recent introduction of the pathogen
289 into an area. Often, one genotype is dominant in the introduced populations and a limited
290 number of mutations explain the minor variation among genotypes (Harrington *et al.*, 2003).
291 Similarly, in our results, one haplotype of Group I represented 26 out of 31 isolates, and the
292 other haplotypes could be considered as variants of haplotype 1. Though few SNPs were
293 identified, the presence of SNP sites in a population could be effectively used for detection purposes. A
294 SNP site could be identified by an appropriate restriction endonuclease whose recognition sequence has
295 been altered or introduced by the SNP. In combination with a PCR assay, the corresponding SNP can
296 be analyzed as a cleaved amplified polymorphic sequence (CAPS) marker (Thiel *et al.*, 2004). The
297 costs of a CAPS assay is generally low, especially when it relies on commonly used restriction
298 enzymes. Our results are partially in accordance with previous results obtained by using the VCG
299 analysis on other isolates of wild and cultivated rocket (Garibaldi *et al.,* 2006): two VCGs were found
300 and, based on complementation to known testers, they were identified as *formae speciales conglutinans*
301 and *raphani* (Catti *et al.,* 2007). In other studies, where the same isolates were analysed for IGS
302 sequence and VCG groups, isolates associated with the same VCG had identical sequences and
303 clustered together (Fourie *et al*., 2009).

304 The *F. oxysporum* isolates examined differed widely in disease severity index. The *F.*
305 *oxysporum* isolates were weakly, moderate or highly pathogenic on rocket plants. These results agree
306 with previous studies, which showed variation in the virulence of *F. oxysporum* isolates from rocket
307 (Garibaldi *et al.,* 2006; Catti *et al.,* 2007), lamb's lettuce (Gilardi *et al.,* 2008) and onion (Dissanayake
308 *et al.,* 2009). Some of the variation in the aggressiveness found in the isolates of the *forma specialis*
309 *raphani* cannot be easily linked to the IGS sequencing results. Several explanations are possible for
310 the lack of association between isolate aggressiveness and genetic markers. First, the
311 genetic markers used, such as IGS sequences, may not be linked to aggressiveness traits.
312 According to Pariaud et al. (2009), aggressiveness is determined by the combination of
313 numerous quantitative traits, and there can be significant differences in aggressiveness

314 among isolates that belong to the same genetic group as defined by neutral markers. In a
315 recent study on *Ceratocystis fimbriata* (Harrington et al. 2011), for example, a large variation in
316 aggressiveness was found within and among pathogen populations from different hosts, but
317 aggressiveness was not correlated with genetic markers. Secondly, the genetic markers used in
318 our study may not be linked to the genes that define aggressiveness, making it difficult to associate a
319 definite phenotype to the genotypes. Since aggressiveness components are defined by quantitative
320 traits, variations in several of these components will occur in natural populations through mutations
321 (Pariaud et al. 2009).

322 Phylogenetic analysis based on IGS sequences revealed also the presence of isolates with a
323 different origin. The isolates used in the study fell into four phylogenetic groups (I to IV). Nakimi *et al.*
324 (1994) reported that genetic differences between the two groups of the *forma specialis melonis* which
325 permitted identification of two different IGS groups could be due to geographic isolation followed by
326 their rapid dispersal throughout the world. Later, Appel and Gordon (1995) identified 13 IGS
327 haplotypes among a population of 56 *F. oxysporum* isolates collected in Maryland and California.
328 Alves-Santos *et al.* (1999) found 6 different IGS haplotypes among 128 isolates of *F. oxysporum.*
329 According to them, the diversity of the IGS haplotype within *F. oxysporum* suggests that sexual
330 reproduction is infrequent or absent in this fungus. Recently, Dissanayake *et al.* (2009) reported four
331 different clusters among 30 isolates of *F. oxysporum* on onion in Japan.

332 Interestingly, by considering the pathogenicity on wild and cultivated rocket of the strains
333 included in Group I, belonging to the *forma specialis raphani*, a partial host specialization could be
334 observed between pathogen isolates from different hosts and their original hosts. In particular, the
335 average disease index of the isolates from *D. tenuifolia* was higher on *D. tenuifolia,* and the average
336 disease index of the isolates from *E. vesicaria* was higher on *E. vesicaria*. Moreover, isolates from
337 cultivated rocket showed, on average, a higher degree of aggressiveness compared to the isolates from
338 wild rocket.

339 By considering the strains present in Group II and III, there are two isolates coming from
340 rocket and three strains belonging to the *forma specialis matthioli*. The two groups are phylogenetically
341 distant. The isolates and strains of the two groups tested showed a highly variable virulence level: FR
342 20/03 was medium to highly virulent, FR 4A/02 showed low to medium virulence, ATCC16602 was
343 not pathogenic or low virulent, while ATCC16601 showed low to high virulence. Due to the low

344 number of strains used in the current study no generalization can be done, but the *forma specialis*
345 *matthioli* seems formed by two genetically different population with different virulence behavior, and
346 such characteristics should be better elucidated by future studies.

347 Three isolates (FR-10B/02, FR-15A/02 and FR-17/03) from rocket were not pathogenic or
348 moderately virulent on cultivated or wild rocket. These isolates were not similar to the other strains
349 tested. The IGS analysis placed these isolates in Group IV and their sequences were very close to *F.*
350 *oxysporum* f. sp. *spinaciae*. These isolates may have been pathogenic on previously planted crops in
351 the same field. Given the fact that presently over 80 *formae speciales* have been described, an
352 enormous number of potential hosts and cultivars would have to be used for conclusive identification
353 (Fravel *et al.,* 2003). Although putatively not pathogenic strains have been described, and some have
354 been employed successfully as biocontrol agents to suppress soilborne pathogens (Larkin *et al.,* 1996;
355 Fuchs *et al.,* 1997; Spadaro and Gullino, 2005; Olivain *et al.,* 2006), the null hypothesis that some
356 isolates may be not pathogenic is virtually impossible to test given the huge number of potential
357 vascular plant hosts (O' Donnell *et al.,* 2009). Recently Ma *et al.* (2010) sequenced a strain of
358 *Fusarium oxysporum* f. sp. *lycopersici* (FOL) and demonstrated that the transfer of lineage-specific
359 chromosomes between strains of *F. oxysporum*, was able to convert a not pathogenic strain into a
360 pathogen. Transfer of LS chromosomes between otherwise genetically isolated strains explains the
361 polyphyletic origin of host specificity and the emergence of new pathogenic lineages in *F. oxysporum.*
362 The not pathogenic strains can be easily converted to pathogenic ones by transferring lineage-specific
363 genes (van der Does *et al*., 2008) or even chromosomes. Moreover, other factors, such as symbiosis
364 with bacteria could explain the pathogenic and not pathogenic feature of some strains of *Fusarium*
365 *oxysporum* (Minerdi *et al*., 2008). Ecto-symbiotic bacteria are capable of silencing the expression of
366 genes involved in fungal pathogenesis, changing in turn the characteristics of hyphae. This
367 phenomenon may result from a direct response to bacterial substances.

368 The IGS sequence analysis confirmed to be a precise and reproducible tool to evaluate genetic
369 similarity or identity and can separate *formae speciales* of *F. oxysporum.* Anyway, recent papers
370 demonstrated the utility of using multiple gene sequencing inside the FOSC. The widespread
371 genealogical discordance between the IGS rDNA and EF-1α bipartitions recently reported (O'Donnell
372 *et al*., 2009), provides argument against using single-locus data for phylogenetic reconstruction and for
373 inferring species limits within the Fungi (Taylor *et al*., 2000). Although the IGS rDNA has become one

374 of the most popular loci for investigating genetic diversity within the FOSC and its high levels of
375 nucleotide diversity provide a high degree of discriminatory power useful for isolate identification,
376 sometimes the evolutionary history of this locus could obscure an accurate phylogenetic relationships
377 within this species. Future research should involve the sequencing of more genes in order to more
378 accurately understand the phylogeny of some of the isolates used in this study. Anyway, the present
379 findings could provide basic information for breeding rocket resistant to Fusarium wilt disease and to
380 establish appropriate disease control strategies.

381

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389

390 **References**

391 Alves-Santos, F.M., Benito, E.P., Eslava, A.P., & Diazminguez, J.M. (1999). Genetic diversity of
392 *Fusarium oxysporum* strains from common bean fields in Spain. *Applied Environmental*
393 *Microbiology, 65,* 3335-3340.

394 Amatulli, M.T., Spadaro, D., Gullino, M.L., & Garibaldi, A. (2010). Molecular identification of
395 *Fusarium* spp. associated with bakanae disease of rice and assessment of their virulence. *Plant*
396 *Pathology, 59*, 839-844.

397 Appel, D.J., & Gordon, T.R. (1995). Intra-specific variation within populations of *Fusarium*
398 *oxysporum* based on RFLP analysis of the intergenic spacer region of the rDNA. *Experimental*
399 *Mycology, 19,* 120-128.

400 Appel, D.J., & Gordon T.R. (1996). Relationships among pathogenic and non-pathogenic isolates of
401 *Fusarium oxysporum* based on the partial sequences of the intergenic spacer region of the
402 ribosomal DNA. *Molecular Plant-Microbe Interaction, 9,* 125-138.

403 Catti, A., Pasquali, M., Ghiringhelli, D., Garibaldi, A., & Gullino, M.L. (2007). Analysis of vegetative
404 compatibility groups of *Fusarium oxysporum* from *Eruca vesicaria* and *Diplotaxis tenuifolia*.
405 *Journal of Phytopathology, 155,* 61-64.
406 Chatterjee C., & Rai J.N. (1974). Fusarium wilt of *Eruca vesicaria* – observation on comparative
407 pathogenicity of some strains of *Fusarium oxysporum*. *Indian Phytopathology, 28,* 309–311.
408 Dissanayake, M.L.M.C., Kashima, R., Tanaka, S., & Ito, S.I. (2009). Genetic diversity and
409 pathogenicity of *Fusarium oxysporum* isolated from wilted Welsh onion in Japan. *Journal of*
410 *General Plant Pathology, 75,* 125-130.
411 Edel, V., Steinberg, C., Avelange, I., Laguerre, G., & Alabouvette, C. (1995). Comparison of three
412 molecular methods for the characterisation of *Fusarium oxysporum* strains. *Phytopathology, 85,*
413 579-585.
414 Engelbrecht, C.J.B., Harrington, T.C., Alfenas, A.C., & Suarez, C. (2007). Genetic variation in
415 populations of the cacao wilt pathogen, *Ceratocystis cacaofunesta*. *Plant Pathology, 56,* 923-
416 933.
417 Enya, J., Togawa, M., Takeuchi, T., Yoshida, S., Tsushima, S., Arie, T., & Sakai, T. (2008). Biological
418 and phylogenetic characterization of *Fusarium oxysporum* complex, which causes yellows on
419 *Brassica* spp. and proposal of *F. oxysporum* f. sp. *rapae*, a novel *forma specialis* pathogenic on
420 *B. rapa* in Japan. *Phytopathology, 98,* 475-483.
421 Fourie, G., Steenkamp, E.T., Gordon, T.R., Viljoen A. (2009) Evolutionary Relationships among the
422 *Fusarium oxysporum* f. sp. *cubense* Vegetative Compatibility Groups. *Applied and*
423 *Environmental Microbiology, 75,* 4770-4781.
424 Fravel, D., Olivain, C., & Alabouvette, C. (2003). *Fusarium oxysporum* and its biocontrol. *New*
425 *Phytologist, 157,* 4770-4781.
426 Fuchs, J.G., Moënne-Loccoz, Y., & Défago, G. (1997). Non-pathogenic *Fusarium oxysporum* strain
427 Fo47 induces resistance to Fusarium wilt in tomato. *Plant Disease, 81,* 492-496.
428 Fujinaga, M., Ogiso, H., Shinohara, H., Tsushima, S., Nishimura, N., Togawa, M., Saito, H., & Nozue,
429 M. (2005). Phylogenetic relationships between the lettuce root rot pathogen *Fusarium*
430 *oxysporum* f. sp. *lactucae* races 1, 2 and 3 based on the sequence of the intergenic spacer region
431 of its ribosomal DNA. *Journal of General Plant Pathology, 71(6),* 402-407.

432 Garibaldi, A., Gilardi, G., & Gullino, M.L. (2002). First report of *Fusarium oxysporum* on lettuce in
433 Europe. *Plant Disease, 86,* 1052.
434 Garibaldi, A., Gilardi, G., & Gullino, M.L. (2003). First report of *Fusarium oxysporum* on *Eruca*
435 *vesicaria* and *Diplotaxis* sp. in Europe. *Plant Disease, 87,* 201.
436 Garibaldi, A., Gilardi, G., & Gullino, M.L. (2004). First report of *Fusarium oxysporum* causing
437 vascular wilt of lamb's lettuce (*Valerianella olitoria*) in Italy. *Plant Disease, 88,* 83.
438 Garibaldi, A., Gilardi, G., & Gullino, M.L. (2006). Evidence for an expanded host range of *Fusarium*
439 *oxysporum* f. sp. *raphani*. *Phytoparasitica, 34,* 115-121.
440 Gilardi, G., Sendhilvel, V., Garibaldi, A., & Gullino, M.L. (2008). Lamb's lettuce (*Valerianella*
441 *olitoria*): new host of *Fusarium oxysporum* f. sp. *conglutinans*. *Journal of Plant Diseases and*
442 *Protection, 115(5),* 229-233.
443 Gupta, A.K. (1988). Fusarium wilt of taramira – a new record for Rajasthan. *Indian Journal of*
444 *Mycology and Plant Pathology, 172,* 239–240.
445 Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *Journal of Molecular*
446 *Biology, 166,* 557-580.
447 Harrington, T.C., Steimel, J., Workneh F., & Yang, X.B. (2003). Characterization of two races of
448 *Phialophora gregata* in North-Central United States. *Phytopathology, 93*, 901-912.
449 Harrington, T.C., Thorpe, D. J., & Alfenas, A.C. (2011). Genetic variation in aggressiveness to native
450 and exotic hosts among Brazilian populations of *Ceratocystis fimbriata*. *Phytopathology, 101*,
451 555-566.
452 Katan, T. (1999). Current status of vegetability groups in *Fusarium oxysporum. Phytoparasitica, 27,*
453 51–64.
454 Kawabe, M., Katsube, K., Yoshida, T., Arie, T., & Tsuchiya, K. (2007). Genetic diversity of *Fusarium*
455 *oxysporum* f. sp. *spinaciae* in Japan based on phylogenetic analyses of rDNA-IGS and MAT1
456 sequences. *Journal of General Plant Pathology, 73,* 353-359.
457 Kawabe, M., Kobayashi, Y., Okada, G., Yamaguchi, I., Teraoka, T., & Arie, T. (2005). Three
458 evolutionary lineages of tomato wilt pathogen, *Fusarium oxysporum* f. sp. *lycopersici*, based on
459 sequences of IGS, MAT1 and pg1, are each composed of isolates of a single mating type and a
460 single or closely related vegetative compatibility group. *Journal of General Plant Pathology,*
461 *71,* 263-272.

462 Kimura, M.A. (1980). Simple method for estimating evolutionary rates of base substitutions through
463 comparative studies of nucleotide sequences. *Journal of Molecular Evolution, 16,* 111-120.

464 Komada, H. (1975). Development of a selective medium for quantitative isolation of *Fusarium*
465 *oxysporum* from natural soil. *Review of Plant Protection Research, 8,* 114-125.

466 Larkin, R.P., Hopkins, D.L., & Martin, F.N. (1996). Suppression of Fusarium wilt of watermelon by
467 non-pathogenic *Fusarium oxysporum* and other microorganisms recovered from a disease-
468 suppressive soil. *Phytopathology, 86,* 812-819.

469 Lievens B., Rep M., & Thomma, B.P.H.J. (2008). Recent developments in the molecular
470 discrimination of *formae speciales* of *Fusarium oxysporum*. *Pest Management Science 64:* 781-
471 788.

472 Ma, L.J., van der Does, H.C., Borkovich, K.A., Coleman, J.J., Daboussi, M.J., Pietro, A.D., et al.
473 (2010). Comparative genomics reveals mobile pathogenicity chromosomes in *Fusarium*.
474 *Nature, 464,* 367-373.

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

478 Minerdi, D., Moretti, M., Gilardi, G., Barberio, C., Gullino, M.L., & Garibaldi, A. (2008). Bacterial
479 ectosymbionts and virulence silencing in a *Fusarium oxysporum* strain. *Environmental*
480 *Microbiology, 10,* 1725-1741.

481 Namiki, F., Shiomi, T., Kayamura, T., & Tsuge, T. (1994). Characterization of the *formae speciales* of
482 *Fusarium oxysporum* causing wilts of Cucurbits by DNA fingerprinting with nuclear repetitive
483 DNA sequences. *Applied Environmental Microbiology, 60,* 2684-2691.

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

487 Olivain, C., Humbert, C., Nahalkova, J., Fatehi, J., L'Haridon, F.L., & Alabouvette, C. (2006).
488 Colonization of tomato root by pathogenic and non-pathogenic *Fusarium oxysporum* strains
489 inoculated together and separately into the soil. *Applied Environmental Microbiology, 72,* 1523-
490 1531.

491 Padulosi, S., & Pignone, D., eds. (1997). Rocket: a Mediterranean Crop for the World. Report of a
492 Workshop, 13–14 December 1996, Legnano, Italy. Rome: International Plant Genetic Resources
493 Institute.
494 Pariaud, B., Ravigne, V., Halkett, F., Goyeau, H., Carlier, J., & Lannou, C. (2009). Aggressiveness and
495 its role in the adaptation of plant pathogens. *Plant Pathology, 58*, 409-424.
496 Pasquali, M., Saravanakumar, D., Gullino, M.L., & Garibaldi, A. (2008). Sequence-specific amplified
497 polymorphism (SSAP) technique to analyse *Fusarium oxysporum* f. sp. *lactucae* VCG 0300
498 isolate from lettuce. *Journal of Plant Pathology, 90(3),* 527-535.
499 Saitou, N., & Nei, M. (1987). The neighbour-joining method: a new method for reconstructing
500 phylogenetic trees. *Molecular Biology and Evolution, 4,* 406-425.
501 Santamaria, P., Elia, A., & Serio, F. (2002). Effect of solution nitrogen concentration on yield, leaf
502 element content and water and nitrogen use efficiency of three hydroponically grown rocket
503 salad genotypes. *Journal of Plant Nutrition, 25,* 245–258.
504 Srinivasan, K., Gilardi, G., Spadaro, D., Garibaldi, A., & Gullino, M.L. (2010). Molecular
505 characterization through IGS sequencing of *formae speciales* of *Fusarium oxysporum*
506 pathogenic on lamb's lettuce. *Phytopathologia Mediterranea, 49,* 309-320.
507 Spadaro, D., & Gullino, M.L. (2005). Improving the efficacy of biocontrol agents against soilborne
508 pathogens. *Crop Protection, 24,* 601-613.
509 Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics
510 Analysis (MEGA) software, version 40. *Molecular Biology and Evolution, 24,* 1596-1599.
511 Taylor, J.W., Jacobson, D.J., Kroken, S., Kasuga, T., Geiser, D.M., Hibbett, D.S., & Fisher, M.C.
512 (2000). Phylogenetic species recognition and species concepts in fungi. *Fungal Genetics and*
513 *Biology, 31*, 21-32.
514 Thiel, T., Kota, R., Grosse, I., Stein N., & Graner, A. (2004). SNP2CAPS: a SNP and INDEL analysis
515 tool for CAPS marker development. *Nucleic Acids Research, 32,* e1-e5.
516 Thompson, J.D., Higgins, D.C., & Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of
517 progressive multiple sequence alignment through sequence weighting, position specific gap
518 penalties and weight matrix choice. *Nucleic Acids Research, 22,* 4673-4680.

519 van der Does, H.C., Lievens, L., Claes, L., Houterman, P.M., Cornelissen, B.J.C., & Rep, M. (2008).
520 The presence of a virulence locus discriminates *Fusarium oxysporum* isolates causing tomato
521 wilt from other isolates. *Environmental Microbiology, 10,* 1475–1485.
522 Woo, S.L., Zoina, A., Del Sorbo, G., Lorito, M., Nanni, B., Scala, F., et al. (1996). Characterisation of
523 *Fusarium oxysporum* f. sp. *phaseoli* by pathogenic races, VCG, RFLP and RAPD.
524 *Phytopathology, 86,* 966-973.
525

526 **Fig. 1** Phylogenetic relationships of 53 isolates. Evolutionary history was inferred using the
527 Neighbour-Joining method. The optimal tree of 511 most parsimonious trees (length = 261) is shown.
528 The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test
529 (10000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the
530 same units as those of the evolutionary distances used to infer the tree. Evolutionary distances were
531 computed using the Maximum Composite Likelihood method and are in the units of the number of
532 base substitutions per site. All positions containing gaps and missing data were eliminated from the
533 dataset (Complete delete option). There were a total of 472 positions in the final dataset. Phylogenetic
534 analyses were conducted using MEGA4.