



DEGLI STUDI DI TORINO

UNIVERSITÀ

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

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

This is the author's manuscript

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/89779 since 2016-10-18T12:36:49Z

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)



UNIVERSITÀ DEGLI STUDI DI TORINO

The final publication is available at Springer via http://dx.doi.org/10.1007/s12600-011-0207-z

9	Genetic diversity and pathogenicity of <i>Fusarium oxysporum</i> isolated from wilted
10	rocket plants in Italy
11	
12	Krishnamoorthy Srinivasan, Davide Spadaro*, Anna Poli, Giovanna Gilardi, Maria Lodovica
13	Gullino and Angelo Garibaldi
14	
15	Agroinnova - Centre of Competence for the Innovation in the Agro-Environmental Sector, University
16	of Torino, Via Leonardo da Vinci 44, 10095 Grugliasco, Turin, Italy.
17	
18	*Corresponding author: Davide Spadaro, E-mail: <u>davide.spadaro@unito.it</u> , Phone: +39-011-6708942,
19	Fax: +39-011-6709307
20	

21 Abstract Thirty-six isolates of Fusarium oxysporum originated from Eruca vesicaria and 22 Diplotaxis tenuifolia together with eight reference strains belonging to the formae speciales raphani, 23 matthioli and conglutinans, typical on the Brassicaceae family, were tested for pathogenicity on two 24 species of rocket plants (E. vesicaria L., syn. E. sativa, cv. 'Rucola coltivata' and D. tenuifolia cv. 25 Winter) cultivated in glasshouse. The results showed that different isolates were slightly, moderately or 26 highly virulent. The strains were examined for differences in the nucleotide sequence of the ribosomal 27 DNA (rDNA) intergenic spacer (IGS) region, about 2.5 kb long. The phylogenetic (neighbor-joining) 28 analysis performed on the isolates permitted to identify four different groups, named I, II, III and IV. 29 Thirty-one isolates out of thirty-six clustered in group I and were genetically similar to F. oxysporum f. 30 sp. raphani. By considering the pathogenicity of the strains included in Group I, a partial host 31 specialization could be observed: the average disease index of the isolates from D. tenuifolia was 32 higher on wild rocket, while the average disease index of the isolates from E. vesicaria was higher on 33 cultivated rocket. Moreover, isolates from cultivated rocket showed, on average, a higher degree of 34 aggressiveness compared to the isolates from wild rocket. About group I, the sequence analysis 35 confirmed the homogeneity of the population, with only 5 parsimony informative SNPs and 5 36 haplotypes. Twenty-six out of 31 isolates belonged to haplotype 1. Group II and III were genetically 37 similar to strains of F. oxysporum f.sp. matthioli. Three other strains, not pathogenic or with medium 38 level of virulence clustered together in Group 4, but their sequence was distant from that of other 39 formae speciales. The pathogenicity and IGS analysis confirmed the presence of virulence variation 40 and genetic diversity among the F. oxysporum isolates studied. To our knowledge, this is the first 41 report of differentiation of *formae speciales* of *F. oxysporum* on rocket plants by IGS analysis.

42

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

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.

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

With over 80 putatively plant host-specific *formae speciales* described (Katan, 1999), members of the *Fusarium oxysporum species complex* (FOSC) collectively represent the most commonly encountered and economically important species complex within the *Fusarium* genus. The *formae speciales* of *F. oxysporum* can be differentiated by pathogenicity tests with appropriate host and by determining the vegetative compatibility groups (VCGs) obtained from heterokaryon formation
between anastomosing nitrate non-utilizing (*nit*) mutants (Garibaldi *et al.*, 2006; Catti *et al.*, 2007). The
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-1 α 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).

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

96

- 97 Materials and methods
- 98

F. oxysporum isolates. Table 1 shows details of the isolates, including their names, year of
isolation, geographical origin and original host. All isolates were maintained on slants of potato
dextrose agar (PDA, Merck, Darmstadt, Germany) at 4°C. The pathogenicity of some of the isolates
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 μ l of a solution of Proteinase K (10 mg ml⁻¹) and 10 μ l of 134 RNAse A (12 mg ml⁻¹) to the lysis buffer in each tube. Purified DNAs were stored at 4°C.

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.

About 5 ml of LB medium containing the antibiotics described for plates were inoculated with a single transforming colony and incubated at 37°C for 16 h. Plasmids were purified from bacterial cells following the manufacturers instruction (Qiagen) and sequenced by the BMR Genomics Centre (Padova, Italy) using the ABI PRISM 3730X1 DNA Sequencer. The complete sequence of the IGS region required the use of additional internal primers and sequencing was performed in both directions (Mbofung *et al.*, 2007). The IGS sequences obtained were deposited in GenBank (accession numbers 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.

Thirty-one isolates (Figure 1) collected from rocket in Italy from 2002 to 2006 as well as *F. oxysporum* f. sp. *raphani* ATCC58110 and ATCC16601, *F. oxysporum* 6-MYA3041 and the reference 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.

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

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

The reference strains ATCC16600, ATCC52557 and ATCC58385 belonging to the *forma* specialis conglutinans formed a distinct group separated from the other isolates, with a bootstrap value of 58%.

257

258 Discussion

259

Eruca vesicaria (cultivated rocket) and *Diplotaxis* spp. (wild rocket) are two members of the Brassicaceae family. Three *formae speciales* of *Fusarium oxysporum* (*raphani, conglutinans* and *matthioli*) are known as being pathogenic on the species of the Brassicaceae family. After the first Fusarium wilts, caused on cultivated and wild rocket by *F. oxysporum*, we created a collection of 36 isolates, originated from 2002 to 2006. By sequencing the ribosomal region IGS, we could effectively differentiate the three *formae speciales* of *F. oxysporum* attacking the Brassicaceae and we could 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 $1-\alpha$ 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.

Our phylogenetic analysis suggests that the strains of *F. oxysporum* f. sp. *raphani* were phylogenetically distinct from the other isolates and are a quite homogeneous population. The results indicate that the genetic diversity in the IGS sequences was extremely low within the same cluster. Thirty-one pathogenic *F. oxysporum* f. sp. *raphani* isolates fell into Group I, consistently supported by bootstrapping of 92%. The high level of homogeneity and the relatively low number of parsimony 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.

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

By considering the strains present in Group II and III, there are two isolates coming from rocket and three strains belonging to the *forma specialis matthioli*. The two groups are phylogenetically distant. The isolates and strains of the two groups tested showed a highly variable virulence level: FR 20/03 was medium to highly virulent, FR 4A/02 showed low to medium virulence, ATCC16602 was 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.

The IGS sequence analysis confirmed to be a precise and reproducible tool to evaluate genetic similarity or identity and can separate *formae speciales* of *F. oxysporum*. Anyway, recent papers demonstrated the utility of using multiple gene sequencing inside the FOSC. The widespread genealogical discordance between the IGS rDNA and EF-1 α bipartitions recently reported (O'Donnell *et al.*, 2009), provides argument against using single-locus data for phylogenetic reconstruction and for inferring species limits within the Fungi (Taylor *et al.*, 2000). Although the IGS rDNA has become one of the most popular loci for investigating genetic diversity within the FOSC and its high levels of nucleotide diversity provide a high degree of discriminatory power useful for isolate identification, sometimes the evolutionary history of this locus could obscure an accurate phylogenetic relationships within this species. Future research should involve the sequencing of more genes in order to more accurately understand the phylogeny of some of the isolates used in this study. Anyway, the present findings could provide basic information for breeding rocket resistant to Fusarium wilt disease and to establish appropriate disease control strategies.

381

Acknowledgements We gratefully acknowledge the financial support from the projects "Advanced Diagnostic Tools, Disease Detection Networking and Technical Extension (ADVANET) for not conventional disease control strategies in vegetable production in Piedmont" and "Safe Food Control Development of innovative systems and technologies for the production, storage, processing and valorization of Piedmontese fruit and vegetables" funded by the Piedmont Region. We especially thank the recently passed away Dr. Robert Milne for his last accurate revision of manuscripts of our research group.

389

390 References

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

- Amatulli, M.T., Spadaro, D., Gullino, M.L., & Garibaldi, A. (2010). Molecular identification of
 Fusarium spp. associated with bakanae disease of rice and assessment of their virulence. *Plant Pathology*, 59, 839-844.
- Appel, D.J., & Gordon, T.R. (1995). Intra-specific variation within populations of *Fusarium oxysporum* based on RFLP analysis of the intergenic spacer region of the rDNA. *Experimental Mycology*, *19*, 120-128.
- Appel, D.J., & Gordon T.R. (1996). Relationships among pathogenic and non-pathogenic isolates of
 Fusarium oxysporum based on the partial sequences of the intergenic spacer region of the
 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.
- Edel, V., Steinberg, C., Avelange, I., Laguerre, G., & Alabouvette, C. (1995). Comparison of three
 molecular methods for the characterisation of *Fusarium oxysporum* strains. *Phytopathology*, *85*,
 579-585.
- Engelbrecht, C.J.B., Harrington, T.C., Alfenas, A.C., & Suarez, C. (2007). Genetic variation in
 populations of the cacao wilt pathogen, *Ceratocystis cacaofunesta*. *Plant Pathology*, *56*, 923933.
- Enya, J., Togawa, M., Takeuchi, T., Yoshida, S., Tsushima, S., Arie, T., & Sakai, T. (2008). Biological
 and phylogenetic characterization of *Fusarium oxysporum* complex, which causes yellows on *Brassica* spp. and proposal of *F. oxysporum* f. sp. *rapae*, a novel *forma specialis* pathogenic on *B. rapa* in Japan. *Phytopathology*, *98*, 475-483.
- Fourie, G., Steenkamp, E.T., Gordon, T.R., Viljoen A. (2009) Evolutionary Relationships among the *Fusarium oxysporum* f. sp. *cubense* Vegetative Compatibility Groups. *Applied and Environmental Microbiology*, 75, 4770-4781.
- 424 Fravel, D., Olivain, C., & Alabouvette, C. (2003). *Fusarium oxysporum* and its biocontrol. *New*425 *Phytologist*, 157, 4770-4781.
- Fuchs, J.G., Moënne-Loccoz, Y., & Défago, G. (1997). Non-pathogenic *Fusarium oxysporum* strain
 Fo47 induces resistance to Fusarium wilt in tomato. *Plant Disease*, *81*, 492-496.
- Fujinaga, M., Ogiso, H., Shinohara, H., Tsushima, S., Nishimura, N., Togawa, M., Saito, H., & Nozue,
 M. (2005). Phylogenetic relationships between the lettuce root rot pathogen *Fusarium 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.

- Garibaldi, A., Gilardi, G., & Gullino, M.L. (2002). First report of *Fusarium oxysporum* on lettuce in
 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.
- Garibaldi, A., Gilardi, G., & Gullino, M.L. (2006). Evidence for an expanded host range of *Fusarium oxysporum* f. sp. *raphani*. *Phytoparasitica*, *34*, 115-121.
- Gilardi, G., Sendhilvel, V., Garibaldi, A., & Gullino, M.L. (2008). Lamb's lettuce (Valerianella *olitoria*): new host of *Fusarium oxysporum* f. sp. conglutinans. Journal of Plant Diseases and
 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.
- Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *Journal of Molecular Biology*, *166*, 557-580.
- Harrington, T.C., Steimel, J., Workneh F., & Yang, X.B. (2003). Characterization of two races of *Phialophora gregata* in North-Central United States. *Phytopathology*, *93*, 901-912.
- Harrington, T.C., Thorpe, D. J., & Alfenas, A.C. (2011). Genetic variation in aggressiveness to native
 and exotic hosts among Brazilian populations of *Ceratocystis fimbriata*. *Phytopathology*, *101*,
 555-566.
- 452 Katan, T. (1999). Current status of vegetability groups in *Fusarium oxysporum. Phytoparasitica*, 27,
 453 51–64.
- Kawabe, M., Katsube, K., Yoshida, T., Arie, T., & Tsuchiya, K. (2007). Genetic diversity of *Fusarium oxysporum* f. sp. *spinaciae* in Japan based on phylogenetic analyses of rDNA-IGS and MAT1
 sequences. *Journal of General Plant Pathology*, *73*, 353-359.
- Kawabe, M., Kobayashi, Y., Okada, G., Yamaguchi, I., Teraoka, T., & Arie, T. (2005). Three
 evolutionary lineages of tomato wilt pathogen, *Fusarium oxysporum* f. sp. *lycopersici*, based on
 sequences of IGS, MAT1 and pg1, are each composed of isolates of a single mating type and a
 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.
- Komada, H. (1975). Development of a selective medium for quantitative isolation of *Fusarium oxysporum* from natural soil. *Review of Plant Protection Research*, 8, 114-125.
- Larkin, R.P., Hopkins, D.L., & Martin, F.N. (1996). Suppression of Fusarium wilt of watermelon by
 non-pathogenic *Fusarium oxysporum* and other microorganisms recovered from a diseasesuppressive soil. *Phytopathology*, *86*, 812-819.
- Lievens B., Rep M., & Thomma, B.P.H.J. (2008). Recent developments in the molecular
 discrimination of *formae speciales* of *Fusarium oxysporum*. *Pest Management Science* 64: 781788.
- 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.
- Namiki, F., Shiomi, T., Kayamura, T., & Tsuge, T. (1994). Characterization of the *formae speciales* of
 Fusarium oxysporum causing wilts of Cucurbits by DNA fingerprinting with nuclear repetitive
 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 two485 locus DNA sequence database for typing plant and human pathogens within *Fusarium*486 *oxysporum* species complex. *Fungal Genetics and Biology*, *46*, 936-948.
- Olivain, C., Humbert, C., Nahalkova, J., Fatehi, J., L'Haridon, F.L., & Alabouvette, C. (2006).
 Colonization of tomato root by pathogenic and non-pathogenic *Fusarium oxysporum* strains
 inoculated together and separately into the soil. *Applied Environmental Microbiology*, *72*, 15231531.

- 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.
- Saitou, N., & Nei, M. (1987). The neighbour-joining method: a new method for reconstructing
 phylogenetic trees. *Molecular Biology and Evolution*, *4*, 406-425.
- Santamaria, P., Elia, A., & Serio, F. (2002). Effect of solution nitrogen concentration on yield, leaf
 element content and water and nitrogen use efficiency of three hydroponically grown rocket
 salad genotypes. *Journal of Plant Nutrition, 25,* 245–258.
- Srinivasan, K., Gilardi, G., Spadaro, D., Garibaldi, A., & Gullino, M.L. (2010). Molecular
 characterization through IGS sequencing of *formae speciales* of *Fusarium oxysporum*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.
- Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics
 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.
- Thompson, J.D., Higgins, D.C., & Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of
 progressive multiple sequence alignment through sequence weighting, position specific gap
 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.