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

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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

Introduction

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

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

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

Materials and methods

***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).

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

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

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

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

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

PCR products were purified using a QIAquick PCR purification kit (Qiagen). They were ligated with a pDrive cloning vector using the Qiagen cloning kit according to the manufacturer's instructions, in 10 µl ligation mixture. After 2 h incubation, 1 - 2 µl ligation mixtures were inoculated in tubes containing Qiagen EZ competent cells, incubated on ice for 5 min, heated at 42°C for 30 sec without shaking, and incubated on ice for 2 min. Two hundred and fifty µl SOC (Super Optimal broth with Catabolite Repression) medium (Hanahan, 1983) were added to the tubes and the contents were directly plated with each transformation mixture onto Luria-Bertani medium (LB) plates containing 100 µg ml^{-1} ampicillin (Merck), 30 µg ml^{-1} kanamycin (Merck), 50 µg ml^{-1} IPTG, and 80 µg ml^{-1} X-Gal at 37°C for 15 - 18 h for blue/white screening of recombinant colonies. The white colonies were screened using M13 forward (GTTTCCCAGTCAGAC) and reverse (AACAGCTATGACCATG) primers to check the presence of inserts. PCR was performed in a mixture containing 60 µM each dATP, dCTP, dGTP and dTTP, 1 µM each primer, 1 Unit Taq DNA polymerase (Qiagen) and 2 µl 10x colorless buffer using whole cells as template. The PCRs were run as described above but decreasing 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 3730Xl 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).

Phylogenetic analysis. The IGS sequences were aligned using the program DNA Baser v2.71.0 (Heracle Software, Lilienthal, Germany). Sequence alignments were manually adjusted to remove mismatches. Phylogenetic analysis was conducted using MEGA version 4 software (Tamura *et al.*, 2007). IGS sequence data were analyzed to determine the relationships by the distance and parsimony methods. Nine IGS sequences of the strains of *F. oxysporum* isolated from other plant species obtained from GenBank database were used as reference sequences and out-groups (Table 1). A distance matrix for the aligned sequences was calculated by using the Kimura two parameter model (1980) and analyzed with the neighbor-joining method (Saitou and Nei, 1987) using the program ClustalW v.1.6 (Thompson *et al.*, 1994), excluding positions with gaps and missing data (complete deletion option). Bootstrap analysis was based on 10,000 re-samplings.

SNP and haplotype identification in the strains of *F. oxysprum* f.sp. *raphani.* The complete IGS sequences of the 31 *F. oxysporum* isolates belonging to Group I were aligned with the 6 reference strains belonging to the *forma specialis raphani*. The SNPs and haplotypes were identified from contig chromatograms using the software Sequencher (Gene Codes, Ann Arbor, MI, USA). The complete analysis included single-base substitutions, single-base and multi-base deletion/insertion. Only parsimony informative SNPs were considered.

Results

Pathogenicity test. Results of the pathogenicity trials carried out on cultivated and wild rocket with 36 isolates of *F. oxysporum* along with 8 reference strains are presented in Table 1. Typical symptoms of Fusarium wilt were first observed 12 to 14 days after inoculation. Symptoms, including stunting and chlorosis of plants, with brown or black streaks in the vascular system, were similar to those described by Garibaldi *et al.* (2003). Out of 36 isolates, 19 showed on cultivated rocket a high level of virulence (disease index between 61% and 100%), 12 a medium level of virulence (disease index between 31% and 60%), 4 a low level of virulence (disease index between 10% and 30%) and one isolate (FR-15A/02) resulted not pathogenic on cultivated rocket. Most of the highly virulent isolates originated from *E. vesicaria*. Among the reference strains, the three *F. oxysporum* f.sp. *raphani* showed a medium level of virulence, ranging from 42% to 56%. Interestingly, the three *F. oxysporum*

f.sp. *conglutinans* were highly virulent, with a disease index ranging from 83% to 100%. A disease index of 15% and 19% was observed for the reference strains of *F. oxysporum* f. sp. *matthioli* race 1 ATCC16602 and race 2 ATCC16603, respectively. When the same 36 isolates were tested on wild rocket, 29 isolates showed a high level of virulence, 5 showed a medium level of virulence, and two (FR 15A/02 and FR 10B/02) were not pathogenic. The three reference *F. oxysporum* f.sp. *conglutinans* were highly or medium virulent, with a disease index ranging from 40% to 92%. The reference strains *F. oxysporum* f. sp. *matthioli* race 1 ATCC16602 and race 2 ATCC16603 were respectively not pathogenic and highly pathogenic on wild rocket. Generally, the isolates were more virulent on wild rocket, so that wild rocket (cv. 'Winter') could be considered more susceptible to Fusarium wilt than cultivated rocket (cv. 'Rucola coltivata'). The result obtained for the two cultivars should be not generalized to the other cultivars of wild or cultivated rocket and wider pathogenicity tests are needed, because there is likely variation in the resistance of different cultivars within each rocket species. *F. oxysporum* was consistently re-isolated from inoculated plants at the end of the experiments. The re-isolation has been performed by using the semi-selective media for *Fusaria* (Komada, 1975) from both symptomless and affected plants (Garibaldi *et al*., 2004). Similar results were observed in all the trials performed.

IGS sequencing and phylogenetic analysis. The PCR reactions resulted in a product varying from 2,493 to 2,655 bp in the isolates tested. The internal primers generated smaller fragments (ranging from 800 to 1000 bp) with the exception of CNL12 used only to amplify the IGS sequence in each isolate. The assembly of the IGS contigs and their alignment provided a sequence in the final data set of 1,472 bp, 134 of them being parsimony-informative. The complete IGS sequence of each isolate was analyzed to infer the phylogenetic relationship using the Neighbor-Joining method (Fig. 1). Four different clusters (Groups I, II, III and IV) were identified. Maximum Parsimony (MP) analysis found the tree length to be 261; the consistency index was 0.912 (0.872), the retention index was 0.933 (0.933) and the composite index was 0.851 (0.814) for all the sites and between parentheses for the 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

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

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

Discussion

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.*

Although bioassays are very effective, they are time-consuming and laborious. Attempts are made to replace them with molecular identification techniques to group isolates (Lievens *et al.,* 2008; Pasquali *et al.,* 2008). To understand the genetic relationship among the *formae speciales of F. oxysporum,* it was useful to sequence the IGS region of several isolates of *F. oxysporum* (Srinivasan *et al.,* 2010). IGS sequence analysis is very precise and reproducible to evaluate genetic similarity or identity and can separate *formae speciales* and physiological races of *F. oxysporum* better than RAPD and other molecular techniques. Recently, Fujinaga *et al.* (2005) reported that IGS sequences were a useful indicator of physiological races for *F. oxysporum.* The polymorphism of this region was higher than that of other loci, such as the mitochondrial Small Subunit (mtSSU) or the Elongation Factor 1-α (EF) (Mbofung *et al.,* 2007), and allowed the genetic diversity detected by VCG in *Fusarium 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

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

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

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

Three isolates (FR-10B/02, FR-15A/02 and FR-17/03) from rocket were not pathogenic or moderately virulent on cultivated or wild rocket. These isolates were not similar to the other strains tested. The IGS analysis placed these isolates in Group IV and their sequences were very close to *F. oxysporum* f. sp. *spinaciae*. These isolates may have been pathogenic on previously planted crops in the same field. Given the fact that presently over 80 *formae speciales* have been described, an enormous number of potential hosts and cultivars would have to be used for conclusive identification (Fravel *et al.,* 2003). Although putatively not pathogenic strains have been described, and some have been employed successfully as biocontrol agents to suppress soilborne pathogens (Larkin *et al.*, 1996; Fuchs *et al.*, 1997; Spadaro and Gullino, 2005; Olivain *et al.*, 2006), the null hypothesis that some isolates may be not pathogenic is virtually impossible to test given the huge number of potential vascular plant hosts (O' Donnell *et al.*, 2009). Recently Ma *et al.* (2010) sequenced a strain of *Fusarium oxysporum* f. sp. *lycopersici* (FOL) and demonstrated that the transfer of lineage-specific chromosomes between strains of *F. oxysporum*, was able to convert a not pathogenic strain into a pathogen. Transfer of LS chromosomes between otherwise genetically isolated strains explains the polyphyletic origin of host specificity and the emergence of new pathogenic lineages in *F. oxysporum.* The not pathogenic strains can be easily converted to pathogenic ones by transferring lineage-specific genes (van der Does *et al*., 2008) or even chromosomes. Moreover, other factors, such as symbiosis with bacteria could explain the pathogenic and not pathogenic feature of some strains of *Fusarium oxysporum* (Minerdi *et al*., 2008). Ecto-symbiotic bacteria are capable of silencing the expression of genes involved in fungal pathogenesis, changing in turn the characteristics of hyphae. This 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-1α 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.

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