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RESEARCH PAPERS

Fusarium oxysporum f. sp. *echeveriae*, a novel *forma specialis* causing crown and stem rot of *Echeveria agavoides*

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Summary. Symptoms of vascular wilt were observed on several plants of *Echeveria agavoides* cultivated in Italy. Molecular analysis based on the ITS region confirmed the pathogen as *Fusarium oxysporum*. In order to identify the *forma specialis*, thirteen isolates obtained from infected tissues were used for phylogenetic analysis based on four polygalacturonase genes (*Pg1*, *Pg5*, *Pgx1* and *Pgx4*) together with the translation elongation factor 1-alpha (*EF-1alpha*) gene. Sequences generated in this study were aligned with other *formae speciales* of *Fusarium oxysporum* from GenBank to construct phylogenetic trees. Eleven isolates of a new *forma specialis, Fusarium oxysporum* f. sp. *crassulae* described on C*rassula ovata*, were added to the analysis based on *Pg5* and *Pgx1* genes. The results obtained for each genomic region showed that the isolates derived from *E. agavoides* are in a unique group well separated from other *formae speciales* already described. The first report of *F. oxysporum* on *E. agavoides* together with the results obtained in this study suggest a new *forma specialis*, here named *F. oxysporum* f. sp. *echeveriae.*

Keywords: *Fusarium oxysporum* f. sp. *echeveriae,* EF-1α; endopolygalacturonase, exopolygalacturonase.

Introduction

Crown and stem rot on crested molded wax agave (*Echeveria agavoides*), caused by *Fusarium oxysporum,* has been recently described in Italy. This host is a succulent plant belonging to the family Crassulaceae (Garibaldi *et al*. 2013). The affected plants showed extensive chlorosis from the crowns to the stem apices, followed by yellowing and a water-soaked appearance of the stem and leaf tissues. As the disease progresses, the leaves go brown, wilt and then rot. Wilting is initially unilateral, and later affects entire plants. Brown discoloration was observed in the vascular systems of cut stems and leaves. In some cases, the leaves were covered by a whitish-orange mycelium (Garibaldi *et al*. 2013). The disease is currently present in a limited number of nurseries, although it has potential to spread causing significant economic losses due to the increasing cultivation of *E. agavoides* in Italy. A *Fusarium* sp. has been reported as the causal agent of a stem rot on *Echeveria* sp. in the USA (Farr *et al*. 1989).

Fusarium oxysporum is one of the most studied plant pathogens, and Fusarium wilts of vegetable and ornamental crops are very numerous and economically important. Moreover, their management provides considerable challenges to growers (Gullino *et al*. 2012). More than 120 different *formae speciales* of *F. oxysporum* have been described (Armstrong and Armstrong, 1981; O'Donnell and Cigelnik, 1999; Baayen *et al*. 2000; O'Donnell *et al*. 2009; Leslie 2012). Detection and identification of *formae speciales*, classically based on pathogenicity assays (Recorbet *et al*. 2003) is, at present, strongly supported by molecular identification tools (Lievens *et al*. 2012). Based on DNA sequences, several markers have been de-

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veloped in order to identify different *formae speciales* (Baayen *et al*. 2000; Groenewald *et al*. 2006). Genomic regions like the intergenic spacer region (IGS) or Transcription Elongation factor (TEF) are useful but are not able to distinguish between different *formae speciales* for correct identification (O'Donnell *et al*. 2009). The best way to discriminate different *formae speciales* is using a multi-gene phylogenetic approach, with phylogenetic analysis based on different genes (Nirenberg and O'Donnell, 1998; Soltis *et al*. 1999; Lutzoni *et al*. 2004; O'Donnell *et al*. 2004). More recently, polygalacturonase genes were identified as a useful genetic marker (Hirano and Arie, 2009). This enzyme is involved during the pathogenesis process and, in particular, is responsible for plant cell wall degradation, allowing the pathogen to penetrate host tissues (Garcia-Maceira *et al*. 2001).

The aim of the present study was to evaluate whether the *Fusarium oxysporum* responsible for wilt of *E. agavoides* warrants designation as a new *forma specialis*, and to assess population genetic diversity of the pathogen across its known geographic distribution.

Materials and methods

Isolates

Isolates from *E. agavoides* were obtained by plating infected tissues of the host onto Komada's Fusarium-selective medium and incubating at 25°C for 7 d (Komada, 1975). After incubation, the strains were transferred onto potato dextrose agar (PDA, Oxoid) amended with 0.5 mg ml^{-1} streptomycine sulphate, and incubated for 7 d at 25°C. Single-spore cultures were established for each isolate by serial dilution of conidial suspensions by spreading a drop of 10^{-6} and 10^{-8} dilutions was onto PDA medium in a Petri plate. A germinated macroconidium was selected under the microscope and transferred to a new PDA plate. Isolates of *F. oxysporum* f. sp. *crassulae* described by Ortu *et al*. (2013a) were included in the pathogenicity assay (see below). All isolates are listed in Table 1.

DNA extraction

DNA extraction was carried out using the NucleoSpin Plant kit (Macherey-Nagel GmbH and Co.), according to the manufacturer's instructions. For each isolate, fresh mycelium was scraped from the surface of the colony and transferred into a 2 ml tube containing 400 µL of lysis buffer and two tungsten beads (Qiagen Stainless Steel Beads, 5 mm). Mycelium was homogenized using Qiagen TissueLyser for 3 min at 28 repetitions per minute and the lysate obtained was used for DNA extraction. DNA concentration was measured using a NanoDrop spectrophotometer, and the extracted DNA was stored at -20°C until further use.

Pathogenicity assays

Pathogenicity assays were conducted on plants inoculated with 1L of 1×10^7 CFU ml^{-1} conidial suspensions of *F. oxysporum*. These were prepared from 10-d-old cultures grown in potato dextrose broth, that were shaken (90 rpm) at 24°C ± 1 (12 h fluorescent light, 12 h dark). Conidial suspensions were prepared for strains DB 264121 and DB264133 isolated from *Echeveria* and for strains CV2 and CV7 belonging to *F. oxysporum* f. sp. *crassulae* (Ortu *et al*., 2013a) isolated from *Crassula ovata* 'Mini' and 'Magical Three'. Each isolate was inoculated separately onto species belonging to the families Crassulaceae and Cactaceae (Table 4). Plants (four per isolate) were dipped in the conidial suspensions of respective tested strains and then transplanted into pots filled with steam-sterilized substrate (sphagnum peat:perlite:pine bark:clay; 50:20:20:10) and maintained in a glasshouse at 28–33°C. Non-inoculated plants were dipped in sterilized water and used as experimental controls. The severity of *Fusarium* wilt was estimated every 4–7 d after inoculation by counting and removing dead plants, following a method previously reported (Garibaldi, 1966). To confirm the presence of *F. oxysporum*, stem sections of diseased plants were surface-sterilized and plated onto PDA and Komada media and incubated at 25°C for 48–72 h.

PCR amplification

The amplification of *EF-1α*, two exopolygalacturonase genes (*Pgx1* and *Pgx4*) and two endopolygalacturonase genes (*Pg1* and *Pg5*) was performed with the primers reported in Table 2. PCR reactions were performed using a Gene Amp 9700 thermocycler (Applied Biosystem) in a 20 µL reaction mixture containing: 10 ng of gDNA, 1 µL of 10 µM stock (final concentration 0.5 µM) of each primer, 1 unit of *Taq*

Table 1. *Fusarium oxysporum* isolates used in this study, all isolated in Northern Italy.

Strain	Host plant	Source
DB 264121	*Echeveria agavoides*	Isolated from infected plant
DB 264122	*Echeveria agavoides*	Isolated from infected plant
DB 264130	*Echeveria agavoides*	Isolated from infected plant
DB 264131	*Echeveria agavoides*	Isolated from infected plant
DB 264132	*Echeveria agavoides*	Re-isolated from the first pathogen assay
DB 264133	*Echeveria agavoides*	Re-isolated from the first pathogen assay
DB 264134	*Echeveria agavoides*	Re-isolated from the first pathogen assay
DB 264135	*Echeveria agavoides*	Re-isolated from the second pathogen assay
DB 264136	*Echeveria agavoides*	Re-isolated from the second pathogen assay
DB 264137	*Echeveria agavoides*	Re-isolated from the second pathogen assay
DB 264138	*Echeveria agavoides*	Re-isolated from the second pathogen assay
DB 264139	*Echeveria agavoides*	Re-isolated from the second pathogen assay
DB 264140	*Echeveria agavoides*	Re-isolated from the second pathogen assay
CV2	*Crassula ovata* cv. Mini	Ortu *et al.* (2013) a
CV3	*Crassula ovata* cv. Mini	Ortu *et al.* (2013) a
CV7	*Crassula ovata* cv. Magical tree	Ortu *et al.* (2013) a
CV8	*Crassula ovata* cv. Magical tree	Ortu *et al.* (2013) a
CV9	*Crassula ovata* cv. Magical tree	Ortu *et al.* (2013) a
CV11	*Crassula ovata* cv. Magical tree	Ortu *et al.* (2013) a
CV12	*Crassula ovata* cv. Magical tree	Ortu *et al.* (2013) a
CV13	*Crassula ovata* cv. Magical tree	Ortu *et al.* (2013) a
CV14	*Crassula ovata* cv. Magical tree	Ortu *et al.* (2013) a
CV15	*Crassula ovata* cv. Magical tree	Ortu *et al.* (2013) a
CV17	*Crassula ovata* cv. Mini	Ortu *et al.* (2013) a

DNA polymerase (Qiagen), 2 μL of PCR buffer 10×, 1 μL of dNTPs stock (final concentration 0.25 mM), and 0.8 μL of $MgCl_2$ (final concentration 1 mM). The cycling conditions included an initial denaturing step at 94°C for 5 min, followed by 50 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, extension at 72°C for 2 min, and final extension at 72°C for 7 min. A negative control (no template DNA) was included in all experiments. Correct amplifications were verified by electrophoresis on 0.8% agarose gel (Agarose D-1 LOW EEO, Eppendorf). After purification with QIAquick PCR purification kit (Qiagen), PCR products were measured using a NanoDrop spectrophotometer and sent to BMR genomics sequencing service (http://www.bmr-genomics.it/). Sequences were deposited at GenBank, and accession numbers are given in Table 3.

Alignment and phylogenetic analyses

Similarity searches (BLASTN, default parameters) were performed for all sequences. The sequences obtained were used for CLUSTALW multiple sequence alignments through MEGA5 software set to default

Table 2. Primers used to amplify polygalacturonase genes.

Gene	Primer	Nucleotide Sequences (5′→3′)	Source
EF-1α	Ef1	ATGGGTAAGGAAGACAAGAC	O'Donnell *et al.*, 1998
	Ef2	GGAAGTACCAGTGATCATGTT	
Pg1	endoF	CCAGAGTGCCGATACCGATT	Hirano and Arie, 2009
	endoR2	GCTTAGYGAACAKGGAGTG	
Pg5	PG2F	AGATGCAAGGCCGATGATGT	Hirano and Arie, 2009
	PG2R	TCCATGTACTTCTCCTCACC	
Pgx1	PgxF	TCGTGGGGTAAAGCGTGGT	Hirano and Arie, 2009
	PgxR	TTACTATAGGTCGATCAGCC	
Pgx4	exoF2	TTACTGTCCACGAATGAGAAG	Hirano and Arie, 2009
	exoR	ACCCCAACCCCCCTCATCT	

Table 3. Accession numbers of *EF-1α*, *Pg1*, *Pg5*, *Pgx1* and *Pgx4* gene sequences obtained from *Fusarium oxysporum* isolates from *Echeveria agavoides*, and deposited in GenBank database.

Isolate	Accession numbers on GenBank				
	EF-1α	***Pg1***	***Pg5***	***Pgx1***	***Pgx4***
DB 264121	KF372979	KF019117	KF019155	KF019130	KF019143
DB 264122	KF372980	KF019118	KF019156	KF019131	KF019144
DB 264130	KF372981	KF019119	KF019157	KF019132	KF019145
DB 264131	KF372982	KF019120	KF019158	KF019133	KF019146
DB 264132	KF372983	KF019121	KF019159	KF019134	KF019147
DB 264133	KF372984	KF019122	KF019160	KF019135	KF019148
DB 264134	KF372985	KF019123	KF019161	KF019136	KF019149
DB 264135	KF372986	KF019124	KF019162	KF019137	KF019150
DB 264136	KF372987	KF019125	KF019163	KF019138	KF019151
DB 264137	KF372988	KF019126	KF019164	KF019139	KF019152
DB 264138	KF372989	KF019127	KF019165	KF019140	KF030444
DB 264139	KF372990	KF019128	KF019166	KF019141	KF019153
DB 264140	KF372991	KF019129	KF019167	KF019142	KF019154

parameters. Manual corrections were performed for each alignment in order to delete trimmer regions outside and discard incomplete sequences. Phylogenetic trees for each genomic region were constructed in MEGA5 (Tamura *et al*. 2007) using the Neighbour joining method with 1,000 bootstrap repeats with

pairwise deletion option. The evolutionary distances were computed using the Tajima-Nei method and are in the units of the number of base substitutions per site. In each analysis we included sequences derived from different *F. oxysporum formae speciales* obtained from the GenBank database. Recently, a new *forma specialis* of *F. oxysporum* on *Crassula ovata* was described, considering that both species belong to the *Crassulaceae* family on phylogenetic trees based on *Pg5* and *Pgx1* regions, the sequences obtained from *F. oxysporum* f. sp. *crassulae* isolates have been added (Ortu *et al*. 2013a).

RESULTS

Pathogenicity assay

When different plants (*Echinocactus grusonii*, *Echeveria agavoides, Huernia zebrina, Mammillaria ginsamaru, Thelocactus rinconensis, T. bicolor, Crassula ovata* cv. Mini, and *C. ovata* cv. Magical Tree) were inoculated with the strains DB 264121 and DB 264133, only *E. agavoides* showed wilt symptoms, and 4 weeks after inoculation all the inoculated plants had died. On the other hand, when plants were inoculated with *F. oxysporum* f. sp. *crassulae,* only *Crassula ovata* cvs Mini and Magical Tree showed wilt symptoms, whereas *E. agavoides* and the other hosts did not (Table 4).

Phylogenetic analyses

Amplification of the *EF-1α*, *Pg1*, *Pg5*, *Pgx1* and *Pgx4* genes resulted, respectively, in 750, 1560, 1800, 1800 and 1400bp fragments of DNA. The nucleotide sequences were used for alignment and phylogenetic analyses. The results obtained showed that in each phylogenetic tree the isolates derived from *E. agavoides* formed a distinct clade separated from other *formae speciales* of *F. oxysporum,* and also from *F. oxysporum* f. sp. *crassulae* included in the analysis based on *Pg5* and *Pgx1* regions (Figures 1–5).

Discussion

The capability of *Fusarium oxysporum* to specialize on different plant hosts is well known and documented (Gordon and Martyn 1997), and is explained both in relation to host plant occurrence in different geographic areas as well as with the genetic structure of the pathogen. The common presence of different transposable elements (Daboussi and Capy, 2003) on the genome of this pathogen suggests the ability of the fungus for genomic rearrangement in response to selection pressures. In this context, host spread from different countries represents an element of specialization, as well as intensive cultivation of host crop plants.

Table 4. Results of the pathogenicity assays.

Host plant	Family	Percentage of dead plants following inoculation with:				
			Fusarium oxysporum			
		Non inoculated control	DB 264121	DB 264133 (Isolated from *Echeveria agavoides*)	CV2 (Isolated from *Crassula ovata*)	CV7 (Isolated from *Crassula ovata*)
Echinocactus grusonii	Cactaceae	0	0	0	0	0
Echeveria agavoides	Crassulaceae	0	100	100	0	0
Crassula ovata "Mini"	Crassulaceae	0	0	0	100	100
Crassula ovata "Magical Tree"	Crassulaceae	0	0	0	100	100
Mammillaria ginsamaru	Cactaceae	0	0	0	0	0
Thelocactus rinconensis	Cactaceae	0	0	0	0	0
Thelocactus bicolor	Cactaceae	0	0	0	0	0

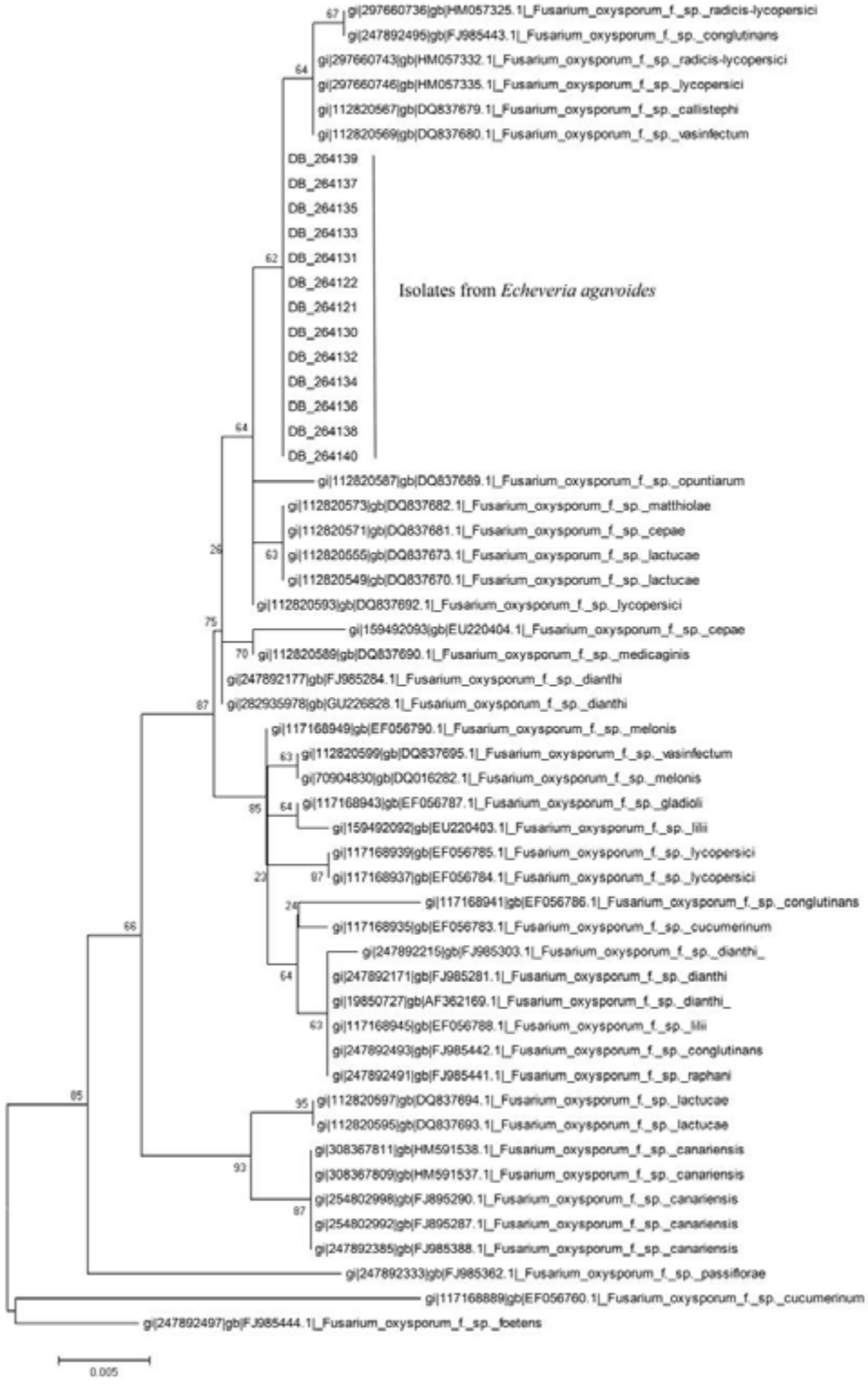

Figure 1. Phylogenetic tree based on *EF-1α* gene sequences, built by Mega5 software with the Neighbor joining method with 1,000 bootstrapping replicates.

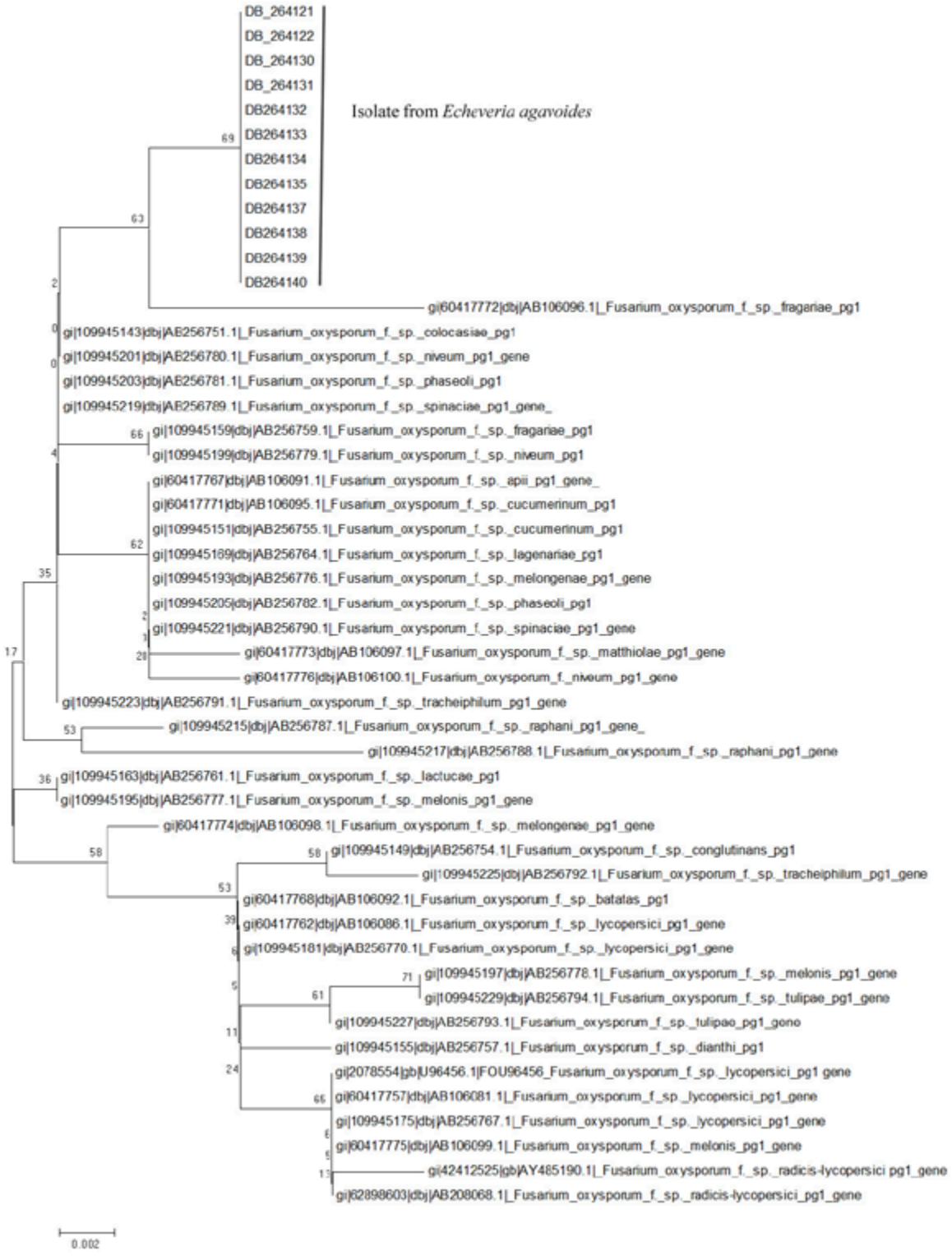

Figure 2. Phylogenetic tree based on *Pg1* gene sequences, built by Mega5 software with the Neighbour joining method with 1,000 bootstrapping replicates.

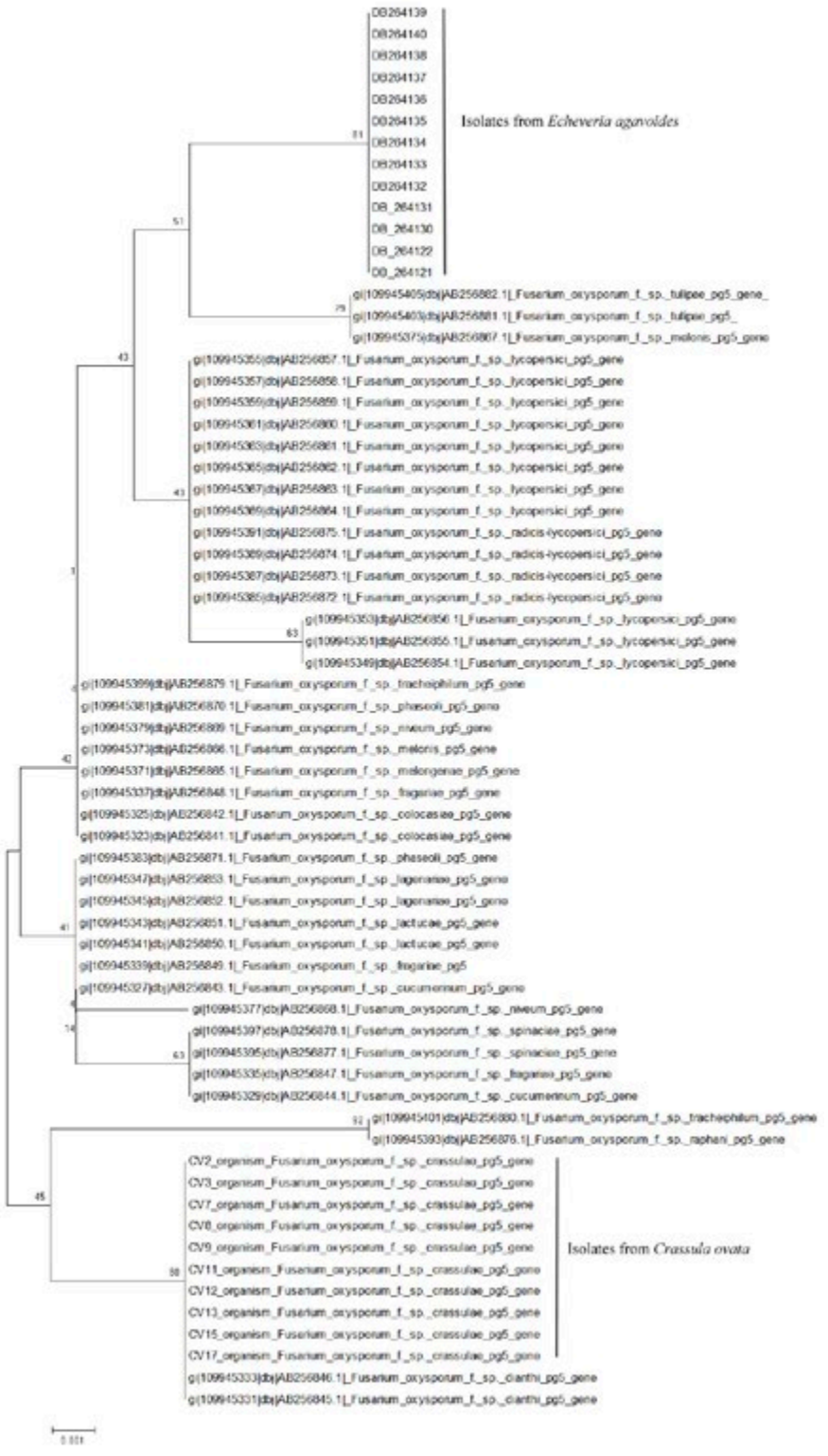

Figure 3. Phylogenetic tree based on *Pg5* gene sequences, built by Mega5 software with the Neighbour joining method with 1,000 bootstrapping replicates.

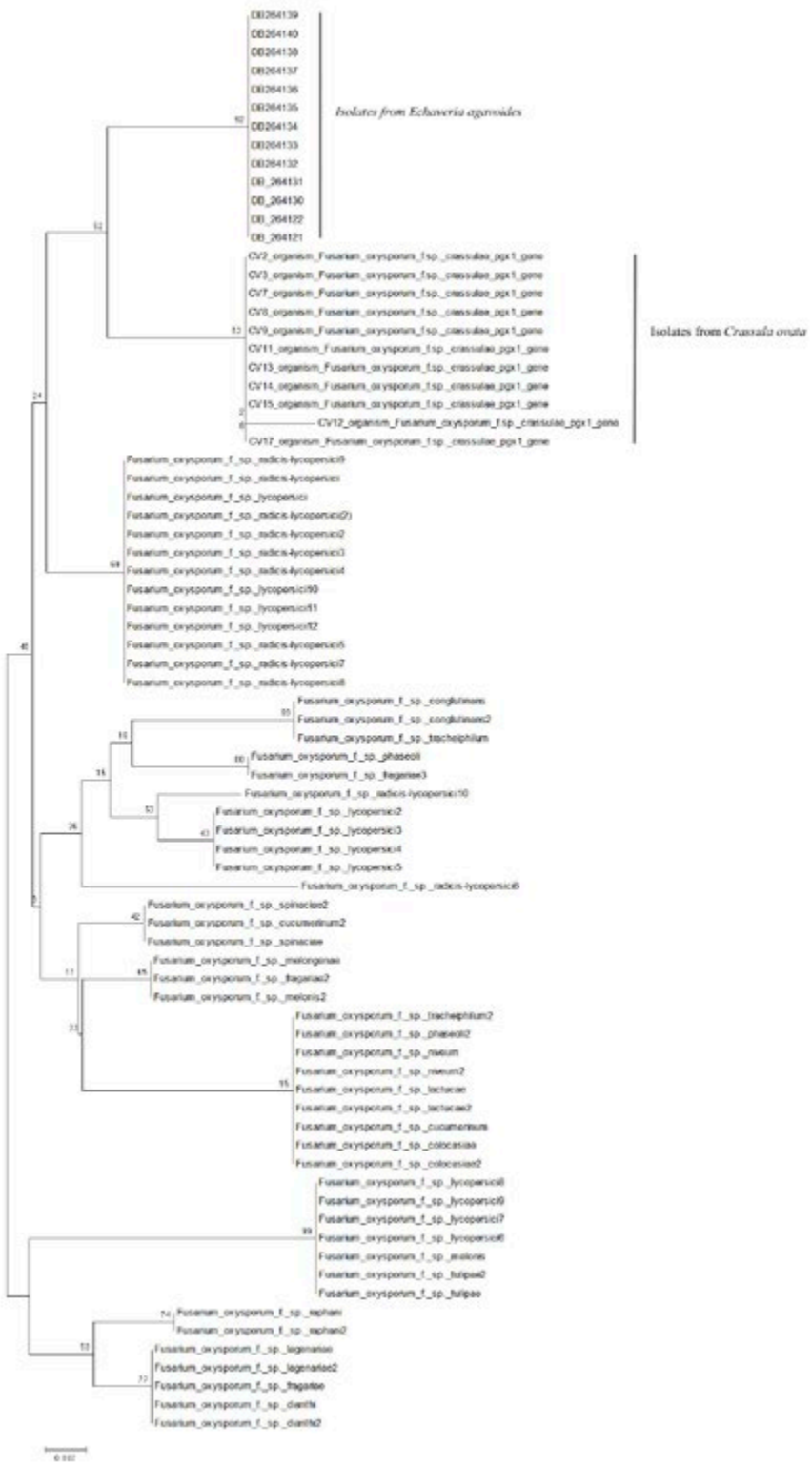

Figure 4. Phylogenetic tree based on *Pgx1* gene sequences, built by Mega5 software with the Neighbour joining method with 1,000 bootstrapping replicates.

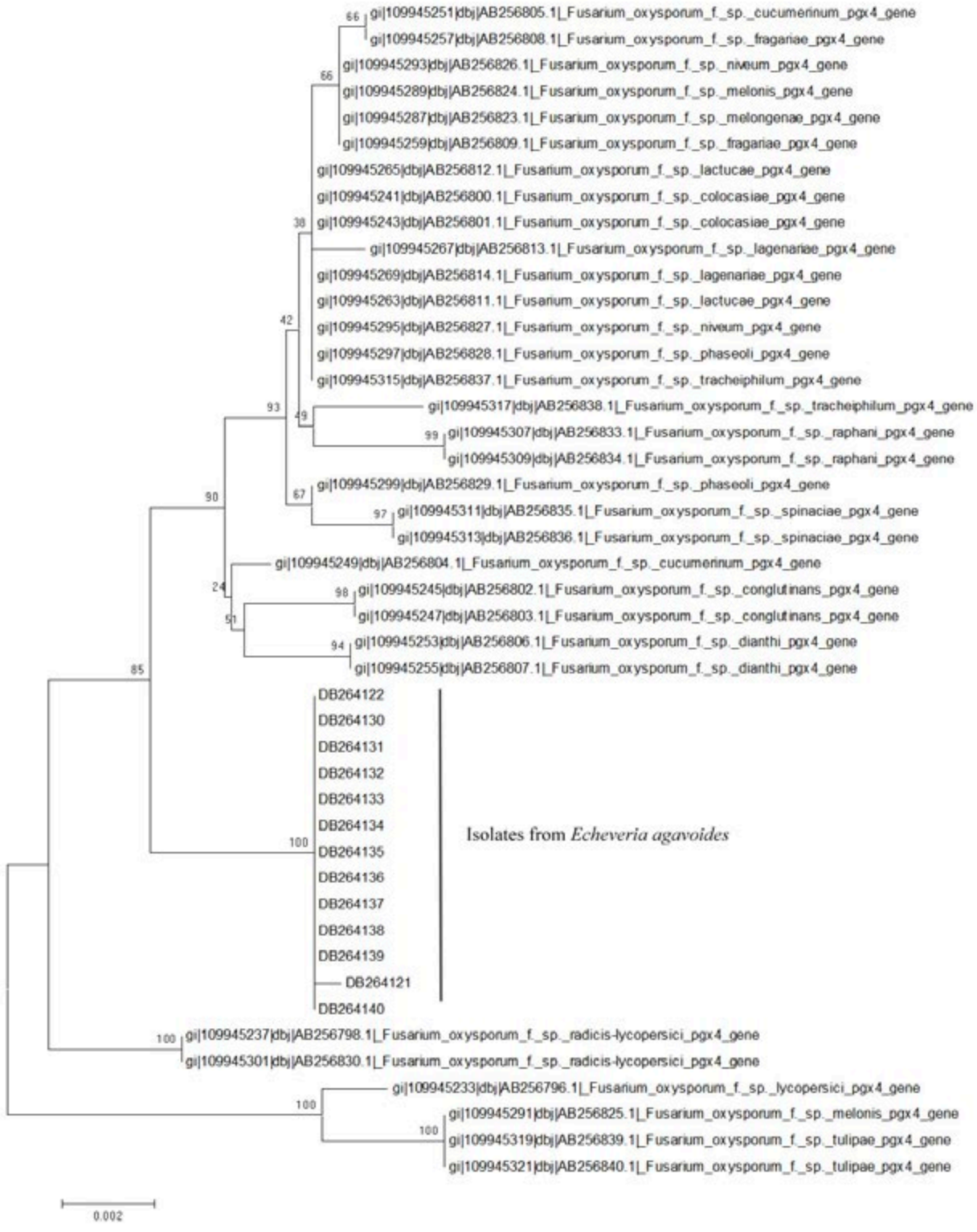

Figure 5. Phylogenetic tree based on *Pgx4* gene sequences, built by Mega5 software with the Neighbour joining method with 1,000 bootstrapping replicates.

A new *forma specialis* of *F. oxysporum* on *Crassula ovata* was recently identified by phylogenetic analysis based on endopolygalacturonase and exopolygalacturonase genes (Ortu *et al*. 2013a). Polygalacturonases are involved during plant infection and, in particular, these enzymes are responsible for plant cell wall degradation by pathogenic hyphae (Carpita and Gibeaut, 1993; Di Pietro *et al*. 2003). Phylogenetic analyses based on these genes have demonstrated the usefulness of these regions for the identification of a *F. oxysporum forma specialis* (Hirano and Arie 2009; Ortu *et al*. 2013a; 2013b). Under our experimental conditions, *F. oxysporum* demonstrated a unique host range causing crown and stem rot of *Echeveria agavoides*, but was not pathogenic to *Echinocactus grusonii*, *Crassula ovata*, *Mammillaria ginsamaru*, *Thelocactus rinconensis* and *T. bicolor*. Moreover, *F. oxysporum* f. sp. *crassulae* and *F. oxysporum* f. sp. *opuntiarum* were also tested on *E. agavoides*, but neither were able to produce disease symptoms. Results obtained by phylogenetic analysis based on *EF-1α*, two genes encoding for two endopolygalacturonases (*Pg1* and *Pg5*) and two genes encoding for two exopolygalacturonases (*Pgx1* and *Pgx4*) confirmed observations in the pathogenicity assays, suggesting the presence of a new *forma specialis*. We propose this to be named *Fusarium oxysporum* Schlechtendal f. sp. *echerveriae* f. sp. nov. Under our experimental condition this new *forma specialis* was able to produce crown and stem rot only onto *Echeveria agavoides* and not on *Echinocactus grusonii, Crassula ovata, Mammillaria ginsamaru, Thelocactus rinconensis* and *T. bicolor.*

An isolate of *F. oxysporum* f. sp. *echerveriae* has been deposited in MycoBank, with the accession number MB809795.

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