CHARACTERIZATION OF *DIAPORTHE HONGKONGENSIS* SPECIES CAUSING STEM-END ROT ON KIWIFRUIT IN TURKEY

I. Erper¹, M. Turkkan², M. Ozcan³, L. Luongo⁴ and A. Belisario⁴

¹ Ondokuz Mayıs University, Faculty of Agriculture, Department of Plant Protection, Samsun, Turkey
² Ordu University, Faculty of Agriculture, Department of Plant Protection, Ordu, Turkey
³ Ondokuz Mayıs University, Faculty of Agriculture, Department of Horticulture, Samsun, Turkey
⁴ Consiglio per la Ricerca in Agricoltura e l’Analisi dell’Economia Agraria (CREA) - Centro di Ricerca Difesa e Certificazione (DC), Via C. G. Bertero 22, 00156 Roma, Italy

SUMMARY

Kiwifruit cultivation is quite recent in Turkey, nevertheless several diseases are becoming dangerous as they reduce fruit production. Among these, stem-end rot is increasingly present in commercial orchards. In autumn 2014, symptoms of stem-end rot of fruit of *Actinidia delicosa* cv. Hayward were observed in three orchards in the Eastern Black Sea region. Morphological, molecular, and pathological analyses confirmed that isolates obtained from diseased kiwifruit were the agents of stem-end rot and belonged to the *Diaporthe lithocarpus/D. hongkongensis* species group. Molecular proximity to the Asian isolates may suggest that the pathogen was introduced in Turkey from East Asia. To our knowledge this is the first report of *D. hongkongensis* on *A. delicosa* in Turkey or elsewhere.

*Keywords*: fruit disease, *Actinidia delicosa*, *Phomopsis*, ITS, TEF.

The Turkish kiwifruit (*Actinidia* spp.) industry is important both nationally and internationally. It makes up only a small part of the Turkish horticulture in general, nevertheless it is profitable and expanding. Kiwifruit (*A. delicosa*) has recently been introduced as a commercial crop in Turkey, covering an area of 2411 ha, from which 41,640 tons of fruits are currently harvested (Anonymous, 2015). The main cultivar planted in commercial orchards is ‘Hayward’ while the cultivation of other species or cultivars is very limited (Atak, 2015). Establishment of kiwifruit plantings has been attempted in several different regions of Turkey, including the humid valleys of the Mediterranean and the Aegean, nevertheless orchards have mainly consolidated in two regions, Black Sea and Marmara (Yildirim et al., 2011). Though kiwifruit cultivation is quite recent in Turkey, several diseases are becoming dangerous since they reduce fruit production. Among these, stem-end rot is increasingly present in commercial orchards.

In autumn 2014, disease symptoms consisting of necrotic and collapsed lesions at the stem-end of fruit of *A. delicosa* cv. Hayward were observed in three orchards in the countryside of Hopa town of Artvin province (Eastern Black Sea region) of Turkey. In particular, on diseased fruit still attached to the plant it was evident the darkening of the brown pubescent skin adjacent to firm healthy tissues (Fig. 1). The brown pubescent skin in the affected end of the fruits becomes soft and lighter in color than the adjacent firm healthy tissues. After peeling the skin, the affected flesh appears water-soaked, disorganized, and light green. External and internal symptoms were similar to those known as stem-end rot caused by *Phomopsis* spp. (Luongo et al., 2011; Koh et al., 2005; Lee et al., 2001) which occurs at the stem-end of the fruit as it ripens.

The aim of the present study was to identify the causal organism of this disease which is having an economic impact on kiwifruit production in Turkey.

For this purpose, 20 diseased kiwifruits directly collected from three kiwifruit orchards were surface-disinfected with sodium hypochlorite (1%) for 1 min. The skin was then peeled and small pieces of flesh (3-5 mm) were excised at the margin of the affected tissue and plated onto potato dextrose agar (PDA) (Oxoid Ltd., UK) medium either not amended or amended with 0.5 g l⁻¹ of streptomycin sulphate (Sigma-Aldrich, USA) (PDAS). Plates were incubated at room temperature and examined daily for mycelial growth. Numerous colonies (about 18) with white aerial mycelium and similar morphology resembling *Phomopsis* spp. were obtained on both media. Subcultures were made onto PDA and incubated for 15 days at 25°C under 12 h light, after which single alpha-conidial cultures were made from sporulating colonies. Four representative single-spore isolates were used for morphological and molecular identification. Measurements of the length and
width of 25 conidia of each conidial type (alpha and beta) for each isolate were made. Morphological studies were based on Gomes et al. (2013). To confirm morphological identification, molecular analysis of four genes, i.e. ITS region of nuclear ribosomal DNA (rDNA), translation elongation factor-1α (TEF-1α), beta-tubulin (TUB), and calmodulin (CAL), was performed. Total DNA was extracted following the protocol of Wizard genomic DNA purification kit (Promega, USA). The quality of genomic DNA was determined by agarose gel electrophoresis and its amount was measured spectrophotometrically (Nanodrop ND-1000 Thermo Fisher Scientific, USA). PCR was performed using a Gene Amp System 9700 thermal cycler (Applied Biosystems, USA). The following primer pairs were used: ITS1/ITS4 to amplify the ITS region (White et al., 1990), EF1-728F/EF1-986R, Bt2a/Bt2b and CAL228F/CAL737R to amplify a partial fragment of TEF-1α (Carbone and Kohn, 1999), TUB (Glass and Donaldson, 1995), CAL (Carbone and Kohn, 1999) genes, respectively. PCR products were purified using NucleoSpin Extract II (Macherey-Nagel, Germany) according to manufacturer’s instructions, then were custom sequenced. The consensus sequences were aligned with additional accessions from GenBank using the BLAST program (http://blast.ncbi.nlm.nih.gov) and multiple sequence alignment was done using CLUSTAL Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Phylogenetic analysis, performed using MEGA version 6.0 software (Tamura et al., 2013), was first conducted on the four single-locus alignments and, successively, the combined alignment of the four loci was analyzed for phylogeny. Multilocus phylogenetic and molecular evolutionary analyses were inferred using maximum likelihood (ML) analysis. The ITS sequences were analyzed alone as well as concatenated with sequences of the other three genes investigated since ITS analyzed alone allowed a wider comparison among isolates from different hosts and geographic origins.

Since fungal isolates were very similar to each other both from morphological and molecular point of view, a pathogenicity test was conducted using only one isolate, KPh-03. The isolate was incubated on PDA at 25°C for 2 weeks to obtain alpha conidia, which were suspended in sterile distilled water, filtered through three layers of sterile cheesecloth, and diluted to a concentration of 1 × 10^6 conidia ml^−1 plus 0.1% polysorbate 20. Inoculations were made on healthy, untreated, nearly ripe kiwifruits of cv. Hayward. Twenty fruits were surface-disinfected with sodium hypochlorite (1%) for 1 min, rinsed in sterile distilled water and dried under a laminar flow hood. Fruits were inoculated either by wounding with a 4-mm-diameter cork-borer and inoculated with a mycelial plug cut from the margin of an actively growing culture, or by injecting 100 μl of the conidial suspension at a depth of 2-3 mm following the method described by Luongo et al. (2011) and Lee et al. (2001) with slight modifications. Controls were inoculated either with sterile distilled water or PDA plugs only. Inoculation points were wrapped with parafilm. Fruits were incubated in a sealed plastic bag at 20-25°C in the dark. The experiment was repeated once.

Alpha conidial colonies produced white cottony aerial mycelial mats with blackish globose to subglobose conidiomata 110-265 μm in diameter (Fig. 1), and creamy conidial drops exuded from the ostioles. Alpha conidia (6.3-8.2 × 2.1-3.1 μm) were unicellular, fusiform, hyaline, biguttulate. Beta conidia (17.5-21.9 × 1.03-1.6 μm) were

Table 1. Species, isolate reference numbers, and accession numbers of the novel sequences deposited in European Nucleotide Archive (ENA) GenBank of Diaporthe hongkongensis isolates obtained from stem-end rot on kiwifruit.

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate No.</th>
<th>GenBank accession No.</th>
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<td></td>
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<td>LT601560</td>
<td>LT601572</td>
<td>LT601568</td>
<td>LT601564</td>
</tr>
</tbody>
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hyaline, aseptate, filiform and curved at one end (Fig. 1). In general, both types of conidia were produced in the same pycnidium, although some pycnidia produced only alpha conidia. On the basis of morphological characteristics the four isolates examined were identified as *Diaporthe lithocarpus* (Gao et al., 2014)/*Diaporthe hongkongensis* (Gomes et al., 2013). Isolates were maintained in PDA slants and in sterile distilled water at 4°C (Burdass and Dorworth, 1994), and were deposited in the culture collection of the Mycology Laboratory of the Plant Protection Department, Faculty of Agriculture, Ondokuz Mayis University (Turkey) with the Nos. OMU-ZRT 2014-KPh01, -02, -03 and -04.

The morphological identification was confirmed by molecular analysis. Based on BLAST results of the ITS region, TEF-1α, TUB and CAL sequence comparison, the similarity ranged from 99 to 100% with *D. lithocarpus* reference strains sequences (99% with KF576285 for ITS, 100% with KF576254 and KF576255 for TEF-1α, 100% with KF576309 for TUB and 99% with KF576232 for CAL). Sequences obtained by PCR amplification of the four genes of isolates OMU-ZRT 2014-KPh01, -02, -03 and -04 were deposited in European Nucleotide Archive (ENA), under the accession numbers listed in Table 1.

Both *D. hongkongensis* and *D. lithocarpus* have been isolated in East Asia, mainly in China (Gao et al., 2014, 2015; Gomes et al., 2013) from Lithocarpus glabra and several additional hosts i.e. camellia, coconut, Dichroa febrifuga, grapevine, and kiwifruit. For the combined analysis of the four genes (ITS region, TEF-1α, TUB and CAL) 12 accessions were retrieved from GenBank (Fig. 2). As to ITS sequence comparison, three accessions of *L. glabra* from China obtained from post-harvest rotten yellow-fleshed kiwifruit of the cv. JinYan were available in GenBank and they were used together with other 12 accessions for phylogenetic comparison (Fig. 3), to a total of 19 nucleotide sequences.

A phylogenetic tree by maximum likelihood (ML) analysis with the combined four gene datasets was inferred based on the Tamura-Nei model. The tree with the highest log likelihood (−5667.8551) is shown in Fig. 2. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter = 0.7859)]. The analysis involved 16 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1,427 positions in the final dataset. The tree was rooted with *Diaporthella corylina*. The four Turkish isolates clearly grouped within the *D. lithocarpus/D. hongkongensis* cluster together with the accessions from China, though they remained closer to each other (Fig. 2), thus confirming the strong morphological similarity already observed. Similar clustering was obtained with ITS sequence comparison by ML analysis based on the Tamura-Nei model, and the tree with the highest log likelihood (−1046.5528) is shown in Fig. 3.
Fig. 3. The procedure was the same as described above. A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter = 0.2749)]. Low morphological and molecular variability was present among the Turkish isolates. Molecular proximity to the Asian isolates may suggest that the pathogen was introduced in Turkey by East Asia. Accessions from India or Puerto Rico showed a certain degree of variability within the D. lithocarpus/D. bongkongensis species group (Fig. 3). At the same time D. cf. baveae2 (CBS681.84) and Diaporthe sp.7 (CBS 458.78) both from India (Gomes et al., 2013) which were very close to the D. lithocarpus/D. bongkongensis in the phylogenetic analysis of Gao et al. (2015) and Gomes et al. (2013) confirmed their proximity either with combined or with the sole ITS analysis (Figs. 2, 3).

As to pathogenicity tests, typical symptoms of stem-end rot appeared on the inoculated kiwifruits within 14 days post inoculation, regardless of the method used. No symptoms occurred on fruits used as control (Fig. 1). Colonies with the same morphology as that of the inoculum were re-isolated from artificially infected fruits, thus fulfilling Koch’s postulates.

With the present study we matched the objective to find the causal organism of kiwifruit stem-end rot in Turkey which is D. lithocarpus/D. bongkongensis. We were unable to make a clear distinction between the two species, though isolates were closer to D. lithocarpus, since they are morphologically and phylogenetically similar in accordance with what stated by Gao et al. (2016) in a recent publication. Actually, since D. bongkongensis was described by Gomes et al. (2013) earlier than D. lithocarpus by Gao et al. (2014), it has the nomenclature priority. Therefore, as stated by Gao et al. (2016), D. lithocarpus should be considered as synonymous of D. bongkongensis. To our knowledge this is the first report of D. bongkongensis on A. delicosa in Turkey or elsewhere.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Roger Cook for his precious scientific suggestions and manuscript revision, and Massimo Galli of CREA-DC for all his technical support.

REFERENCES


