

RAPID COMMUNICATION

Characterization of *Botryosphaeria dothidea* as new pathogen of kiwifruit in Iran

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Vol. 59, No. 1: 134–137, 2019

DOI: 10.24425/jppr.2019.126035

Received: July 30, 2018

Accepted: January 30, 2019

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Abstract

Kiwifruit (*Actinidia deliciosa*) is one of the most significant commercial crops in Iran. In 2015 a destructive disease of kiwifruits was observed in orchards, storage facilities and retail markets, resulting in great economic loss to producers. In this study phenotypic and molecular techniques were applied to characterize the causal agent of kiwifruit rot observed in Mazandaran province, northern Iran. From the similarity among the results of pathogenicity tests, equivalency with standard taxonomic criteria for disease and PCR-based analysis of the ITS region, all the isolates were identified as *Botryosphaeria dothidea*.

Keywords: *Actinidia deliciosa*, *Botryosphaeria dothidea*, ITS, post-harvest, ripe rot

Kiwifruit (*Actinidia deliciosa* (A. Chev.) C.F. Liang et A.R. Ferguson) is a deciduous tree belonging to the Actinidiaceae family. The popularity of kiwifruit is increasing due to its high vitamin C content, balanced nutritional components, minerals, dietary fiber and health-promoting metabolites. Iran is one of the most important producers of the fruit worldwide, followed by Italy, China and New Zealand. The tree is widely planted in northern Iran with a total production of 250×10^3 t annually. Mazandaran province is the main kiwifruit growing area in Iran, with approximately 90% of Iran's total kiwifruit production. So far, several fungi have been reported in association with kiwifruit rot including *Alternaria*, *Colletotrichum*, *Botryosphaeria*, *Cylindrocarpon*, *Phomopsis*, *Phoma*, *Diaporthe*, *Botrytis* and *Penicillium* (Hawthorne and Reid 1982; Opgenorth 1983; Pennycook 1985; Manning *et al.* 2003; Koh *et al.* 2005; Luongo *et al.* 2011; Erper *et al.* 2017). *Botryosphaeriaceae* species, frequently isolated from rotted fruit, is potentially one of the major causal organisms of post-harvest rot of kiwifruit which

is considered to be the most important disease of *A. deliciosa* (Lee *et al.* 1998). Clear external symptoms of ripe rot were sometimes absent from the surface of the fruit, but a portion of the fruit surface was collapsed. Water-soaked flesh tissue could be seen in the sunken area when the skin of the collapsed portion was peeled back. A milky-colored spot appeared in the flesh and developed concentrically as the fruit ripened (Li *et al.* 2017). White mycelia emerge from rotted fruit epidermal tissues and broken skin allows fluid to exude from severely decayed fruit (Zhou *et al.* 2015). To the best of our knowledge, no specific research has determined the phenotype and molecular characteristics of ripe rot of kiwifruit caused by *Botryosphaeria dothidea* in Iran. The aim of this study was to isolate and identify the cause of ripe rot of kiwifruit based on morphology and sequence analysis of the internal transcribed spacer (ITS).

In northern Iran 43 diseased fruits were collected from orchards, storage facilities and retail markets from September to November of 2015 and 2016. They

were disinfected with 0.5% NaClO for 3 min, and then washed three times with sterile distilled water. Small pieces (0.5–1 cm) from the edges of rotten and healthy tissue of diseased fruits were placed on potato dextrose agar (PDA) medium. Pure cultures of the fungus were obtained using single spore cultures. Isolates were grown on prune-extract agar (PA) (Amponsah *et al.* 2008) under black UV light to induce *in vitro* sporulation. Pathogenicity assay using six isolates was done by inoculation of mycelium plugs on wounded fruits which were disinfected with 70% ethanol, in three replications. Three fruits were treated either with sterile distilled water or with sterile mycelial plaque considered to be the control. Inoculated fruits were maintained in a polyethylene bag at the temperature and light regime as described by Koh *et al.* (2003) for 1 week. Re-isolation of the fungus was performed to fulfil Koch's postulates. Total DNA was extracted from seven pure cultures grown in potato dextrose broth after 48 h using the method described by Barnes *et al.* (2001). The polymerase chain reaction mixtures (PCR; 25 µl) contained 1 µl of genomic DNA, 1.25 µl of each primer (10 mM), 17.5 µl of sterile deionized water, 0.5 µl of dNTPs (10 mM), 0.2 µl of Taq DNA polymerase, 0.8 µl of MgCl₂ (50 mM), and 2.5 µl of 10X buffer [200 mM Tris HCl (pH 8.4), 500 mM KCl]. The ITS region was amplified using the primers ITS5 (Forward) 5'-(GGAAGTAAAAGTCGTAACAAGG)-3' and ITS4 (Reverse) 5'-(TCCTCCGCTTATTGATA TGC)-3' (White *et al.* 1990). Amplified sequence was compared with the other sequences found in the National Center for Biotechnological Information (NCBI) database (<http://www.ncbi.nlm.nih.gov>) using Basic Local Alignment Search Tool (BLAST).

Naturally diseased fruits were healthy in appearance, with soft tissues and slightly sunken infected sites. The sunken part under the fruit's skin had water soaked, rotted tissues with a dark-green margin and was milky colored in the center (Figs 1A–B). Based on morphological characters, the fungus was identified as *B. dothidea*. The morphological identification of *B. dothidea* was based primarily on characteristics of the conidial state, normally found in pycnidia and easily cultured *in vitro* (Phillips 2002; Koh *et al.* 2003). The optimum temperature range for mycelial growth was 25–32°C, but growth did not occur under 9°C or over 38°C. The preliminary mycelial colony of the fungus was white, but turned black after 7 days incubation at 25°C. *Globus pycnidia*, 250 × 127 µm in diameter, were produced on PDA 1 month after incubation at 25°C (Fig. 1C). Conidia were hyaline, unicellular and fusoid (Fig. 1D), with a size average of 25–30 × 5.5–7 µm. In pathogenicity assays, disease symptoms appeared as soft lesions with black-green margins and milky centers in fleshy tissue (Fig. 1E). The disease, so-called "ripe rot", has been previously reported as one of the

most important post-harvest diseases of kiwifruit from other countries (Koh *et al.* 2003; Zhou *et al.* 2015; Li *et al.* 2017). The use of ITS5/ITS4 primers caused the amplification of the expected bands in the isolate, which was in accordance with the classification of the morphological features employed in this research. The region sequence of ITS rDNA revealed 99% homology (Fig. 2) to *B. dothidea* isolate HJ20 (HQ328038).

The sequence was deposited in Genbank with accession number MG273743 for *B. dothidea* isolate act. One isolate of the fungus was also deposited in the Iranian Fungal Culture Collection under IRAN 3045C accession number. These observations were in agreement with the reports of Koh *et al.* (2003) and Zhou *et al.* (2015). This is the first report of kiwifruit rot caused by *B. dothidea* in Iran. In conclusion, considering the current problem of ripe rot of kiwifruit and the importance of developing management programs, diagnosis of the pathogens, using morphological and molecular characteristics, is a key step for successful control of kiwifruit decay in the humid region of northern Iran. According to morphological characteristics, phylogenetic analysis and pathogenicity testing applied in this study, *B. dothidea* is the causal agent of the disease. *Botryosphaeria dothidea* is an economically important phytopathogen for different crops. It is able to act as primary or secondary pathogens on kiwifruit under humid conditions (Phillips 2002). Previous studies indicated that a wound is not necessary for the pathogen to infect the fruit since pathogen entry can be via natural openings (Li *et al.* 2006). A wound just enhances the severity of symptoms (Zhou *et al.* 2015). Zhou *et al.* (2015) reported that *B. dothidea* causes shoot blight of kiwifruit, and leaves and shoots may play an important role in the infection cycle. Since the threat of disease is increasing in Iran, due to planting the highly susceptible kiwifruit throughout the region, more studies should be conducted in order to find a rational solution based on the infection cycle of the pathogen in orchards. The importance of the subject results from the fact that many hosts act as powerful repositories of *B. dothidea* inoculum for kiwifruit. It is clear that asexual reproduction is a dominant character for fungus dissemination. The pycnidia and pseudothecia of fungus can overwinter in orchards and produce conidia and ascospores as inoculum resources for fruit and leaves in the following year. Controlling the spread of *B. dothidea* within storage facilities and production sites is difficult because of the presence of inoculum sources as well as the broad host range of the pathogen. In many production areas, fruits show no apparent symptoms before the infection is wide spread. Indeed, fruits which have been treated with protective fungicides may appear healthy until the fungicide efficacy is lost and the pathogen population increases. Overall, storing the fruit at 0–1°C, treatment of the fruits with

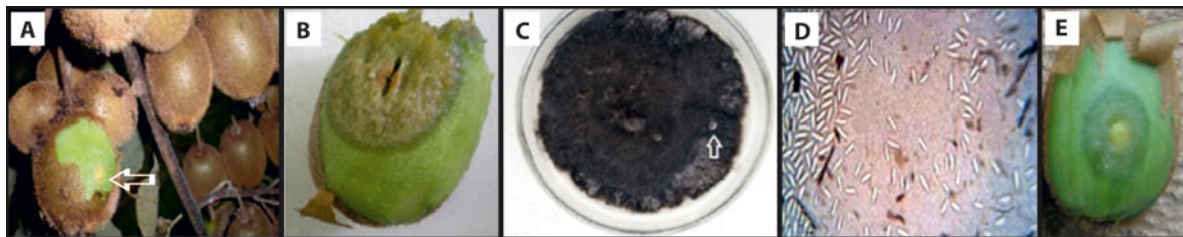


Fig. 1. (A) Initiation of infection (arrow) on kiwifruit in the orchard, (B) symptoms of naturally diseased kiwifruit caused by *Botryosphaeria dothidea*, (C) pycnidia formation of *B. dothidea* on PDA medium (arrow), (D) conidial morphology of *B. dothidea* and (E) symptoms of disease in pathogenicity on kiwifruit

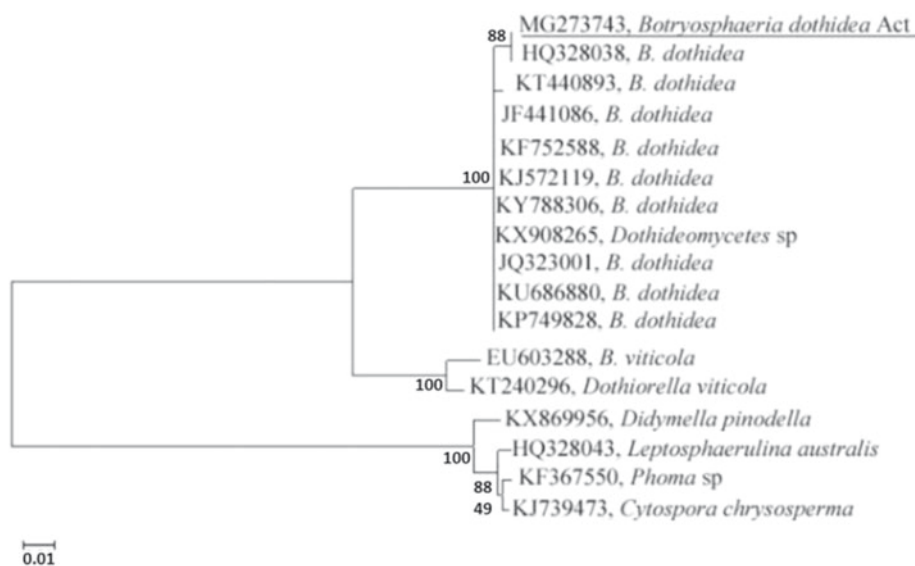


Fig. 2. Dendrogram constructed by the neighbor-joining method showing the phylogenetic relationship (99%) between the Iranian isolate of *Botryosphaeria dothidea* Act (MG273743) and *B. dothidea* isolate HJ20 (HQ328038) based on the sequences generated using ITS5/ITS4 primers

natural compounds to induce resistance, and the use of preventive fungicide before or after fruit harvest, are recommended for disease management (Terry and Joyce 2004; Yao and Tian 2005).

Acknowledgements

This study was part of a project entitled “Kiwifruit gray mold bio-control using native yeasts collected from northern Iran” funded by the Iranian Research Institute of Plant Protection (grant number 2-16-16-93164).

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