Australasian Plant Disease Notes (2018) 13: 15
https://doi.org/10.1007/s13314-018-0301-x

Citrus x *aurantiifolia*, a new host report of *Macrophomina phaseolina* in Iran

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Received: 30 January 2018 / Accepted: 7 May 2018 / Published online: 11 May 2018

Abstract

Charcoal root rot-like symptoms were observed on Mexican lime (*Citrus* x *aurantiifolia*) plants in a nursery located in Hormozgan province, southern Iran. The fungus was identified as *Macrophomina phaseolina* based on morphological and molecular characteristics. Pathogenicity tests revealed the association of six *M. phaseolina* isolates with disease. Reisolation from roots of inoculated plants yielded isolates of *M. phaseolina* with morphological characteristics identical to those of the original isolates used for inoculations, thus fulfilling Koch's postulates. This is the first record of charcoal rot caused by *M. phaseolina* on citrus in Iran.

Keywords Charcoal rot · *Macrophomina phaseolina* · Mexican lime · *Citrus* x *aurantiifolia*

Macrophomina phaseolina is a soil-borne, microsclerote-producing fungus with a worldwide distribution. It causes charcoal rot and ashy stem blight of several important crops including sorghum, sunflower, corn, melon and beans (Frederiksen 1986; Mahdizadeh et al. 2011; Mahmoud and Budak 2011; Olaya et al. 1996; Pearson et al. 1986). *M. phaseolina* is especially prevalent in subtropical and tropical arid climates. This fungus usually infects plants that are subjected to severe stresses induced by drought and high temperatures (Olaya et al. 1996). In Iran, *M. phaseolina* causes significant damage in soybean (Raeyatpanah et al. 2002) and sunflower (Razavi and Pahlavani 2004). During the last decade, many other crops including marigold, cantaloupe, cumin, hemp, mung bean, okra, tomato, turnip, watermelon (Mahdizadeh et al. 2011) and strawberry (Sharifi and Mahdavi 2012) were reported as new hosts for *M. phaseolina*.

In July 2017, charcoal root rot-like symptoms were observed on Mexican lime (*Citrus* x *aurantiifolia*) plants in a nursery located in Hormozgan province, an economically important citrus-producing area of southern Iran. The diseased plants showed symptoms of root rot, leaf yellowing, and premature death. The rotted roots were firm and dark brown to orange brown with numerous black microsclerotia present in the vascular and on cortical tissues. Fruiting bodies or other fungal structures were not observed. The disease generally occurred in discrete patches and its incidence ranged from 10 to 50% within the patches. Two hundred symptomatic root fragments obtained from 20 affected plants were surface-disinfested with 2% sodium hypochlorite solution for 3 min, rinsed three times in sterile distilled water, dried on sterilised paper, and plated onto potato dextrose agar (PDA) supplemented with 150 μg ml^{-1} of streptomycin sulfate. Petri plates were incubated at 30 °C for 6 days with a 12-h photoperiod. In culture, the mycelium was initially hyaline, later becoming grey (Fig. 1), and after 4–5 days of incubation numerous black, spherical-oblong-irregularly shaped microsclerotia, 60–118 × 52–98 μm, developed in the colonies. Pycnidia were not produced by any isolate. The morphology of the fungus was identical to that already described for *M. phaseolina* (Holliday and Punithalingam 1970). A representative isolate was deposited in the fungal culture collection of the Iranian Research Institute of Plant Protection, Tehran, Iran (Accession No. IRAN 3046C). The diseased plants tested negative for *Phytophthora* spp., *Rhizoctonia solani*, *Fusarium* spp. and other pathogens.

To confirm the identification, DNA from the isolate IRAN 3046C was extracted from a lab culture, and the internal transcribed spacer region (ITS1–5.8S-ITS2) was amplified by PCR and sequenced using the universal primers ITS1 and

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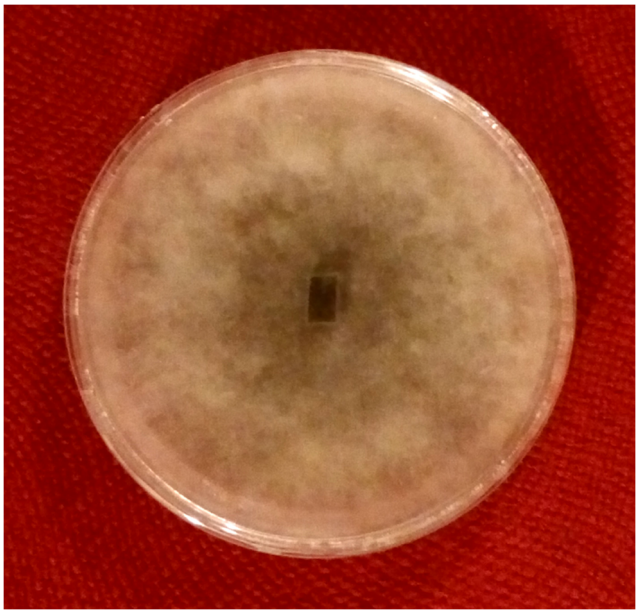

Fig. 1 A four-day-old culture of the isolate IRAN 3046C of *Macrophomina phaseolina*

ITS2 (White et al. 1990). The sequence was deposited in GenBank (Accession No. MG636998). BLASTn search of the sequence showed 100% identity with sequences of *M. phaseolina* (KR012878.1, GU046902.1 and GU046898.1). The base substitution model was implemented using MrModeltest2 (Nylander 2004). To estimate invariant sites, a general time reversible model based on Akaike criterion was included among-site rate heterogeneity (GTR + G + I) in phylogenetic analyses. Phylogenetic relationships and the related tree were constructed using MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003) (Fig. 2). After discarding burn-in (25% of the samples) samples and evaluating convergence, the remaining samples were kept for further analysis. To determine the equilibrium distribution and estimation of the Bayesian posterior probabilities of clades the Markov chain Monte Carlo (MCMC) method within a Bayesian framework was run for 10 million generations (Larget and Simon 1999) using the 50% majority rule. The Bayesian posterior probability values higher than 0.50 are presented on appropriate clades. The phylogenetic analysis was inferred and re-drawn using Dendroscope V.3.2.8 (www-ab.informatik.uni-tuebingen.de/

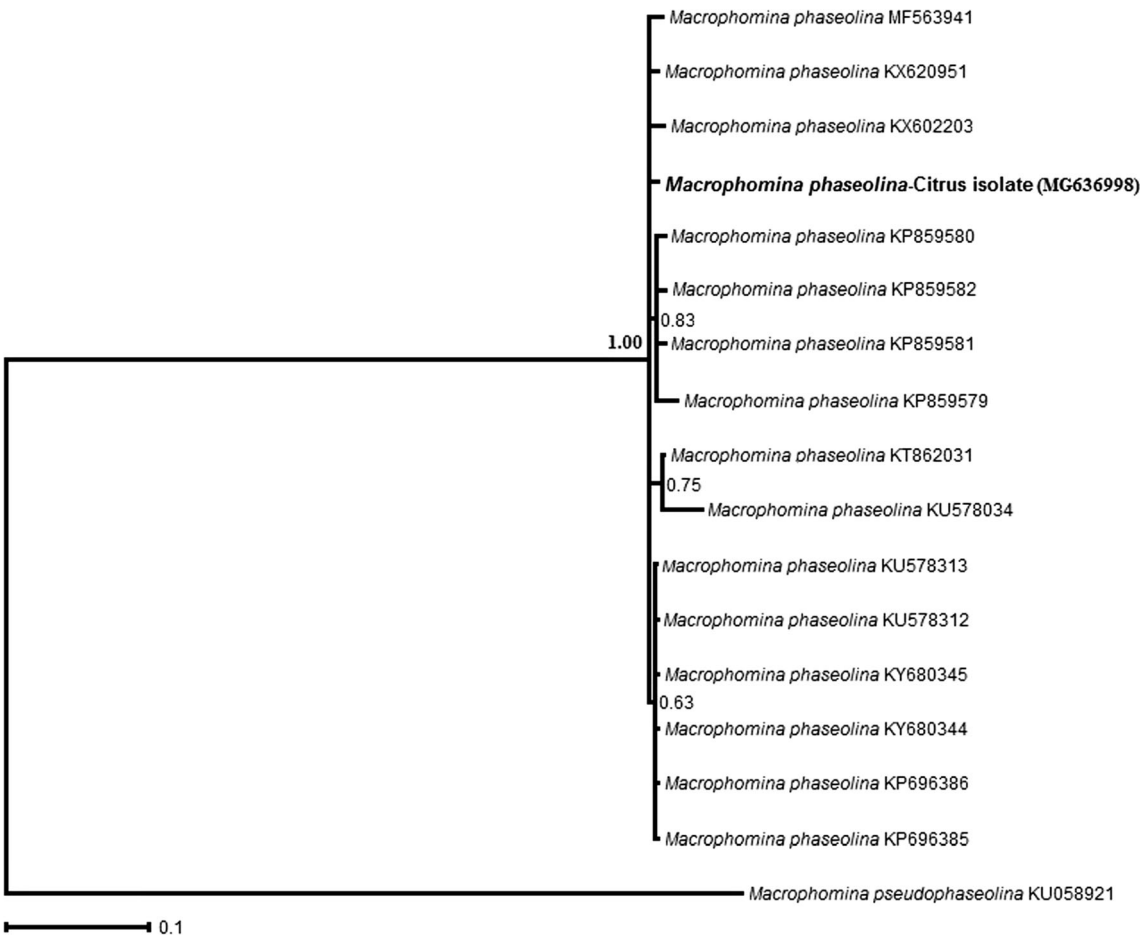

Fig. 2 The 50% majority rule consensus tree inferred from Bayesian analysis of the citrus isolated sequence under the GTR + G + I model. The citrus-isolated sequence is indicated in bold

software/dendroscope) and CorelDRAW version X7, respectively.

Pathogenicity tests, repeated twice, were performed to establish the association of six hyphal-tipped *M. phaseolina* isolates, including the isolate IRAN 3046C, with disease. Each isolate was used to inoculate 70-day-old healthy Mexican lime plants growing in plastic pots containing autoclaved peat/soil mixture for 70 days. Inocula of *M. phaseolina* isolates, consisting of microsclerotia, were obtained free of culture medium by aseptically placing a small, colonised agar block from an actively growing culture in a flask containing sterile potato dextrose broth (PDB). The flask was incubated at room temperature for three months until a thick mat composed predominantly of microsclerotia formed on the surface of the broth. The mat was separated from the medium by vacuum filtration, rinsed three times in sterile distilled water, and dried at 35 °C for 72 h. The dried mycelial mats, consisting mostly of microsclerotia, were then ground with a mortar and pestle and passed through a 325 μm mesh to obtain smaller clumps. Prior to the experiment, the germination of microsclerotia on water agar medium was determined to be 80%. Microsclerotia were mixed with 1000 g of sterile air-dried sand and stored at 4 °C until used for inoculations (Goudarzi et al. 2008). The 70-day-old plants were transplanted into the autoclaved peat/soil mix (5000 g in plastic pots) infested with the microsclerotia/sand mix at the rate of 100 viable microsclerotia g^{-1} soil and maintained in a greenhouse at 30± 2 °C. Ten control plants were transferred to non-infested soil. All plants were watered once a week. After three weeks, all inoculated plants began to show symptoms on leaves and roots, similar to the symptoms of the nursery plants. By five weeks, disease severity ranged from 75 to 100% depending on isolate. Extent of colonisation was rated according to a 1 to 9 scale in which 1 refers to no visible symptoms and no formation of sclerotia, whereas 9 indicates all tissues of the root are colonised and densely covered by sclerotia (Olaya et al. 1996). No symptoms were observed on control plants. Microsclerotia were produced after seven weeks on roots of 85% of the surviving plants. For each isolate tested, *M. phaseolina* was reisolated only from inoculated plants, fulfilling Koch's postulates.

To the best of our knowledge, this is the first record of occurrence of charcoal rot on citrus caused by *M. phaseolina* in Iran. Similarly, charcoal rot of citrus has been reported from Kenya (Kung'u et al. 2002) and India (Chakraborty et al. 2011). Based on the incidence and severity of symptoms, charcoal root rot of citrus, an emerging disease, is considered as a potential threat to citrus industry in southern Iran.

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