Black Spot Disease of Pomegranate Caused by Cladosporium Cladosporioides in China

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Abstract. Pomegranate (*Punica granatum* L.) is an important fruit crop in Yunnan Province, China. Black spot disease was observed on pomegranate fruits in Mengzi County. Symptomatic pomegranate fruits were collected during the autumn of 2012-2016, and used for the isolation of suspected fungus. On the basis of morphological characteristics and nucleotide homology, the isolate was identified as *Cladosporium cladosporioides*. Koch's postulates were performed. This is the first report of *Cladosporium cladosporioides* causing black spot disease of pomegranate in China.

1. Introduction

Pomegranate (*Punica granatum* L.), an economically important species of the tropical and subtropical regions of the world, regarded as a "Fruit of Paradise". Due to its delicious edible fruits, pharmaceutical and ornamental usage, pomegranate is one of the major fruit crops of arid zones. Cultivation of this fruit crop is adversely affected by various diseases, resulting in massive losses to grower. These diseases include: wilt disease caused by *Ceratocystis fimbriata* and *Fusarium oxysporum* (Huang *et al*, 2003, Xu *et al*, 2011); black spot of pomegranate caused by *Alternaria alternate* (Ezra *et al*, 2010); heart rot caused by *Aspergillus niger* (Yehia, 2013); leaf spots caused mainly by *Cercospora punicae* (*Pseudocercospora punicae*), *Alternaria alternate*; fruit rot caused by *Coniella granati*; *Phomopsis aucubicola* and *Phytophthora* sp.; anthracnose caused by *Colletotrichum gleosporoides* (Kishore *et al*, 2011); bacterial blight caused by *Xanthomonas axonopodis* (Benagi *et al*, 2011); crown rot on pomegranate caused by *Coniella granati* (Çeliker *et al*, 2012); dieback and fruit rot caused by *Pilidiella granati* (Mirabolfathy *et al*, 2012); pomegranate Gray Mold caused by *Botrytis cinerea* (Bardas *et al*, 2009); and post-harvest Fruit Rot caused by *Penicillium glabrum* (Spadaro *et al*, 2010). Methods of controlling diseases were researched all over the world.

Pomegranate is an important fruit crop in Yunnan Province of China. Black spot disease was observed in pomegranate orchards in Mengzi County in recent years. Black spot symptoms were observed on pomegranate fruits, which appeared at the late stage of summer on all cultivars, spreading quickly in fall during harvest season. Black spot disease of pomegranates decreases commercial value of pomegranate, because of the unpleasant appearance of the skin. The disease can also decrease pomegranate nutrition value and taste. In severe scenarios the disease can infect the inner parts of the fruits and destroy the wholes fruits.

2. Materials and Methods Results

Symptomatic fruits were collected in autumn 2012-2016. Surfaces were disinfected by placing them in 75% ethanol for 30s, then transferred to a 0.1% aqueous mercuric chloride solution for 120s and rinsed with sterilised water three times. Lesion pieces were placed on potato dextrose agar (PDA) and incubated at 28℃.

The Pathogen was identified based on its morphological characteristics and nucleotide homology sequence analysis. Pathogenicity tests using Koch's postulates.

3. Results

Black spot symptoms on pomegranate range from a single spot to several spots on the fruit surface; black spots are irregular (8-20mm) on fruit (Fig. 1. A, B); black spots on pomegranate can be with or without annulations, and the black area around spots can be either pitted or not pitted. Damage to fruits is mostly limited to the peel surface, but the edible tissue can be damaged when the disease becomes severe (Fig. 1. C). this disease infects pomegranate fruits when they are still on the tree, however most severe disease symptoms are often observed on post-harvest fruits.

Fungal colonies emerging from symptomatic tissue cultured on PDA, flat, velvet, olive-brown or green top view whereas dark brown or nearly black dorsal are viewed. Hyphae light brown, septate, branched, 3.1-3.8μm width; conidiophore 2-4 tufted, upright or slightly curve, dark olive, septate, 2 or

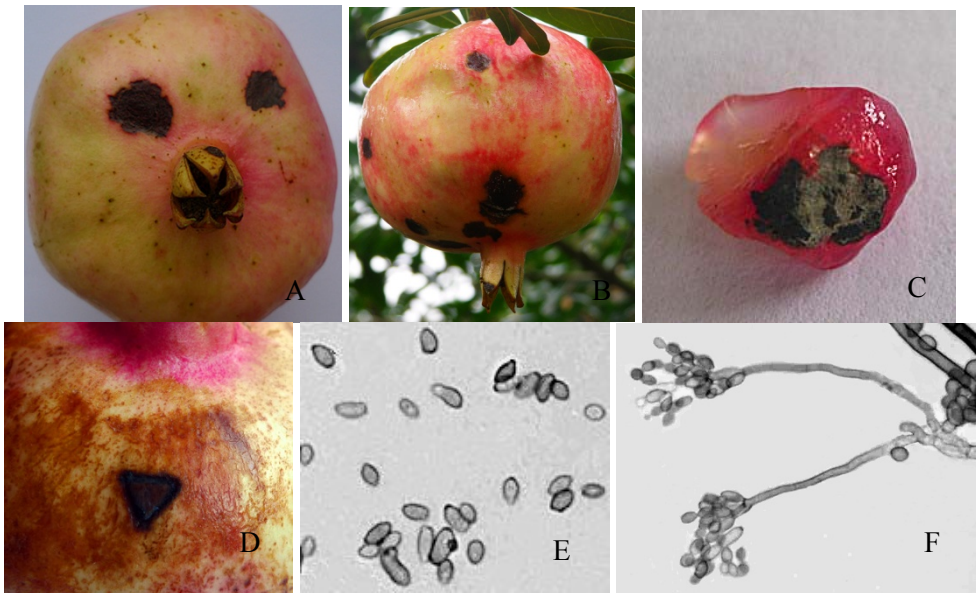

Figure 1. A. B. Black spot disease on fruit. C. Black spot disease infects pomegranate seed. D. Black spot at 10 days after inoculation. E. Ram conidia and conidia. F. Conidiophore and branched chains of conidia.

more obvious cicatrix at the apice, conidiogenous cell and conidiophore together extended; conidia budding at cladosporium, concatenate, prolate ellipsoid-, lemon- or oval-shaped, solitary/pale to olive, surface smooth, non-septate, 5.4-8.1 x 3.2-3.8μm, which had morphology and conidia typical of *Cladosporium* spp; five isolates were obtained.

Genomic DNA of the fungal pathogen was extracted, rDNA ITS region was amplified by PCR, using universal primer ITS1 (ITS1:5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). PCR was conducted as follows: primer ITS1 0.5 μl primer ITS4 0.5 μl, genomic DNA 0.5μl, PCR Mix 12.5μl ddH_2o 11.0μl were mixed to a 25μl total volume. The PCR reaction condition was 94℃, 4min; 36 cycles for 94℃, 1min, 54℃, 1min, 72℃, 1 min; 72℃, 10min; held in 4℃. Following that, 5μl PCR amplification product was mixed with 2μl 6× loading

buffer and electrophoresed in 1.5% agarose gel (120V, 40 min). Subsequently, the expected DNA fragments were purified. Sequence analysis of the rDNA ITS region yielded PCR fragments of 529bp respectively. The ITS sequence was submitted to GenBank (KF134535). Blast analysis of the ITS sequence exhibited 99% similarity with the nucleotide sequences for *Cladosporium cladosporioides* (JQ619815). Blast searches and phylogenetic analysis revealed that the highest similarity for (KF134535) is with *C. cladosporioides* (JQ619815) (Fig.2). On the basis of morphological characteristics and nucleotide homology, the isolate was identified as *Cladosporium cladosporioides*.

Pathogenicity tests using Koch's postulates were performed, the pomegranate surface was disinfected and a triangle wound cut. Conidia were harvested from eight-day-old cultures growing on PDA, adjusted to a suspension of 10^6 conidia/ml and sprayed on wounded parts of pomegranate, while control was treated with sterile water alone. Twenty replicates for the inoculated were used. All fruits were covered with plastic bags to maintain 90% relative humidity and kept in the dark for twenty days at ambient temperature conditions. Although the method of inoculation was somehow artificial, symptoms appeared 7 days after inoculation, and the inoculated fruits developed typical black spot symptoms 10 days later (Fig. 1. D). Characteristic lesions that developed on inoculated fruits were similar in appearance to those diseased fruits in the pomegranate orchards. All controls remained healthy, reisolation from the inoculated tissues consistently yielded *C. cladosporioides*, thus fulfilling Koch's postulates.

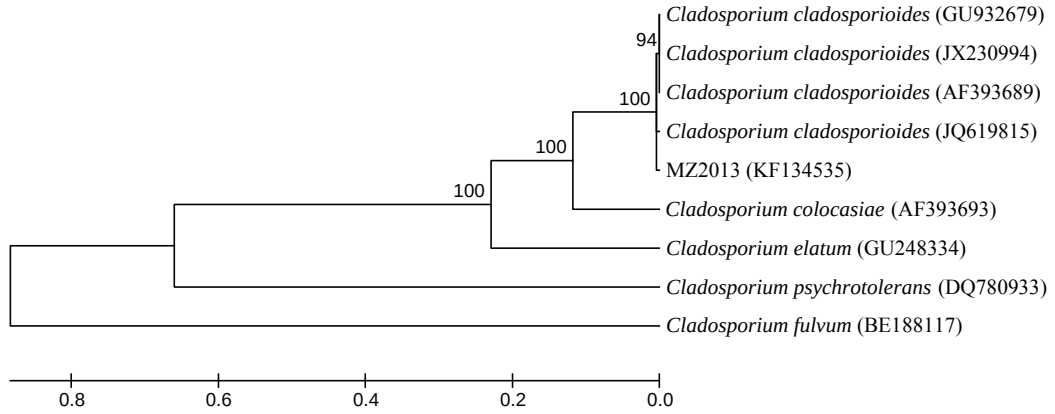

Figure 2. Phylogenetic tree of MZ2013 based on ITS sequence

The phylogenetic tree was generated based on the up mega method using MEGA V.4. The bootstrap values are indicated at each node and were calculated with 1000 repetitions. Cladosporium as out group.

4. Conclusion and discuss

The genus *Cladosporium* is one of the largest genera of *dematiaceous hyphomycetes*, and *C. cladosporioides* is a widely distributed saprophyte reported to occasionally infect the lung, skin, eye and brain of humans. As such, *C. cladosporioides* can infect human cerebrospinal fluid (Kantarcioglu *et al*, 2002) and cause human subcutaneous phaeohyphomycosis (Sood and Makkar, 2000), phaeohyphomycosis (Vieira *et al*, 2001), and can also infect dog causing mycotic encephalitis and nephritis (Poutahidis *et al*, 2009). The growth of *C. cladosporioides* in wounds on cantaloupe rinds facilitates migration of *Salmonella poona* into subsurface mesocarp tissues (Richards and Beuchat, 2005). Recently *C. cladosporioides* was reported as the causal agent of sooty spot of postharvest Satsuma mandarin (Tashiro *et al*, 2013).

Black spot disease of pomegranates, a common disease of pomegranates in the tropics and subtropics. On the basis of morphological characteristics and nucleotide homology sequence analysis, the pathogen was identified as *Cladosporium cladosporioides*. Although *Alternaria* black spot on pomegranate fruit has been reported in Israel (Ezra *et al*, 2010) and leaf spot on pomegranate was previously reported in India (Madhukar and Reddy, 1998), to the best of our knowledge, this is the first report of *Cladosporium cladosporioides* causing black spot disease of pomegranate in china.

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