Morphological and Molecular Characterization of Pomegranate Fruit Rot Pathogen, *Chaetomella raphigera*, and its Virulence Factors

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Abstract A new fungal pathogen was isolated from rotten pomegranates collected from the orchards of different parts of Maharashtra. The pathogen was morphologically identified as *Chaetomella raphigera* followed by sequencing of ITS and D1/D2 hypervariable region of LSU (28S) of rRNA gene. The pathogen produced pectinase, cellulase, xylanase and protease in liquid medium at a concentration of 71, 13.8, 54.3 and 7 U/ml respectively. Enzyme activity was also determined during pathogenesis in the tissues artificially infected by *C. raphigera*. Xylanase activity was maximum (25.1 U/g) followed by pectinase (19.2 U/g) and cellulase (1.5 U/g), whereas, protease activity was unnoticed. There was significant correlation ($P < 0.05$) between disease rating scale and pectinase, xylanase and cellulase activity in infected tissues. This indicates the simultaneous production of hydrolytic enzymes that aids in necrosis of fruit tissues. The elevated levels of these enzymes in infected tissues as compared with control suggest their possible role in pathogenesis. Thus, pectinase, cellulase and xylanase produced by *C. raphigera* acts as major virulence factors in the development of fruit rot in pomegranates. This is a first report of fungal fruit rot caused by *C. raphigera* in pomegranate.

Keywords *Chaetomella raphigera* · Pomegranate · Fruit rot · Virulence factor · ITS sequencing

Pomegranate (*Punica granatum*) is a valuable fruit crop commercially grown in various parts of India and pomegranates from Maharashtra have export value [1]. Fungal fruit spot and fruit rot decrease the market value of fruits. These infections are caused by a host of fungal species belonging to *Alternaria*, *Aspergillus*, *Beltaraniella*, *Cercospora*, *Cladosporium*, *Colletotrichum*, *Curvularia*, *Fusarium*, *Phomopsis*, *Phytophthora*, *Rhizopus*, etc. [2]. Phytopathogenic fungi produce hydrolytic enzymes such as cutinases, cellulases, pectinases, xylanases and proteases that can degrade the plant cell wall components and aids in penetration and spreading of fungal pathogens through the plant tissues. Thus, extracellular cell wall-degrading enzymes (CWDE) act as virulence factors [3]. In view of this background, the objectives of this study were to isolate fungal pathogen of fruit rot, its morphological and molecular identification followed by study of their virulence factors.

Pomegranates with spots and rotting symptoms were collected from different orchards of Pune, Nashik and Ahmednagar districts in Maharashtra. The fungi were isolated from the infected parts on sterile potato dextrose agar (PDA) (Hi-Media) containing streptomycin (50 µg/ml). Pathogenicity test of fungal isolate was performed on healthy pomegranates of Bhagwa and Ganesh varieties as these are largely cultivated in Maharashtra. The fungal pathogens were identified by morphological characteristics determined by slide culture technique [4]. Two fungal pathogens (BF79, BF99) isolated from cv. Bhagwa were morphologically identified as *Chaetomella raphigera*. Pycnidia were dark reddish brown, oval, approximately 200 µm × 300 µm in culture. Pycnidia were bearing cylindrical, septate brown colored setae, up to 60–70 µm long and 2–4 µm broad. The setae were thinner and tapering towards the apex and curved. The Pycnidium

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break opens at raphe, showing the presence of filiform aseptate conidiophores. Conidiophores were branched, arising from all the sides of pycnidia and are more in number at the base, approximately 80–90 μm long and 1–2 μm broad. Conidia were produced apically on conidiophores, aseptate, ellipsoidal with rounded ends, smooth, straight or curved, 10–12 μm $\times$ 2–3 μm . These morphological features are shown in Fig. 1. Morphologically, the coelomycetous genera, *Chaetomella* and *Pilidium* are closely linked. But have differences in terms of morphology and rDNA sequence analysis. Both of them produce dark pycnidia that open by a raphe. They have aseptate, ellipsoidal conidia. The species of *Chaetomella* produce pycnidia on the surface and the pycnidia produced by the species of *Pilidium* extend from inside the substratum, ultimately becoming superficial. A major distinguishing feature is that pycnidia of *Pilidium* are soft and lack setae, whereas in the case of *Chaetomella*, there is presence of setae. There are three regions in the pycnidial wall of *Chaetomella* while that in *Pilidium* there are only two regions. *Chaetomella* shows presence of long and filiform conidiophores. The conidiophores may be branched with

phialidic conidiogenous cells that originate at the apex and each septum. The conidiophores in *Pilidium* are also extended but shorter than those found in *Chaetomella* [5].

The pathogen was also identified using ITS region and LSU gene sequencing [6]. The fungal DNA was extracted and quantitated by Nano drop (ND-1000, Thermo scientific, USA). Amplification of internal transcribed spacer region rDNA was done in Gene Amplifier PCR System 9700 (Perkin Elmer, USA) using domain specific primers, namely ITS1 (5'-tccgtagtgtaacctgcgg-3') and ITS4 (5'-tcctcgttattgatatgc-3') [7]. *Taq* DNA polymerase dye terminator cycle was used for sequencing reactions utilizing dideoxynucleotide chain termination method. ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Applied Biosystems Division, Foster City, CA) was used to carry out sequencing reactions. The primers LR5R (tcctgagggaaactcg), LROR (accgctgaactaagc), LR3R (gtctgaacacggacc) and LR16R (ttcaccctaaactcg) were used [8]. DNA sequencing was carried out using ABI 3730XL Genetic Analyzer.

The analysis of these nucleotide sequences was done using BLAST (Basic Local Alignment Search Tool) at the

Fig. 1 Morphological features of *Chaetomella raphigera*: pycnidium in culture (a), section of pycnidium (b), setae (c) and conidia (d). Pathogenicity test on pomegranate (e). Scale bar, (a, b) = 50 μm ; (c, d) = 10 μm ; (e) = 10 mm

Fig. 2 Phylogenetic relationship of *Chaetomella raphigera* based on ITS1, 5.8S, ITS2 rDNA sequence data. Using the Neighbor-Joining method evolutionary history was derived. Next to the branches is percentage of replicate trees according to bootstrap test (1000 replicates). The evolutionary distances were calculated using the p-distance method and are in the units of the number of transitional differences per site. MEGA5 was used for evolutionary analyses

NCBI website. The sequences were deposited in NCBI Genbank database and the accession numbers for the nucleotide sequences are KF308287 and JX863671. A phylogenetic tree was constructed by Neighbor-Joining method along with different representatives (Fig. 2). Besides sequencing of the ITS region, further sequencing of the hypervariable region, D1/D2 of LSU was also done. BLAST analysis of these two nucleotide sequences revealed close similarities with *C. raphigera*. The phylogenetic analysis exhibit homology with different *Chaetomella* isolates but clustered the two isolates separately. Neighbor-Joining tree shows clustering of different *Chaetomella* species and, BF79 and BF99 isolates making a separate branch. This might indicate novel status of these isolates.

Chaetomella raphigera produced pectinase, cellulase, xylanase and protease with relative activity of 0.55, 0.48, 0.41 and 0.15, respectively as determined on solid media (Fig. 3). The levels of pectinase, cellulase, xylanase and protease in liquid medium were 71, 13.8, 54.3 and 7 U/ml respectively. Enzyme activity was also determined during pathogenesis, i.e., in the tissues artificially infected with *C. raphigera*. In such tissues, the concentration of xylanase (25.1 U/g) was higher than pectinase (19.2 U/g) and cellulase (1.5 U/g), whereas the protease activity was unnoticed. The production of these hydrolytic enzymes in infected tissues and their correlation with disease rating scale is shown in Fig. 3. Disease rating of fungal infection was determined by measuring the diameter of rot around the point of inoculation. The levels of these enzymes were found elevated with increasing disease rating scale. This indicates that xylanase, pectinase and cellulase were

involved in the degradation of the plant tissue. These are the major CWDEs capable of macerating plant tissues on their own. The production of hydrolytic enzymes by plant pathogenic fungi has been demonstrated in several reports. Jat et al. [9] described that *C. gloeosporioides*, a fruit rot pathogen of banana, produces pectinase and cellulase enzymes, that are associated with the development of fruit rot. There was significant correlation ($P < 0.05$) between disease rating scale and pectinase, xylanase and cellulase activity in the infected tissues as determined by Pearson Correlation Coefficient. Significant correlation ($P < 0.05$) was also recorded between the activities of pectinase, cellulase and xylanase. This indicates that simultaneous production of hydrolytic enzymes aids in the necrosis of fruit tissues.

There are four species of *Chaetomella* namely, *C. acutiseta*, *C. circinoseta*, *C. oblonga*, and *C. raphigera*. *C. raphigera* is the most commonly encountered species of this genus. It is isolated from various samples throughout the world but it is more common in warm temperate to tropical regions [10]. The market value and export cost of pomegranates decreases due to several bacterial and fungal infections [1]. Generally farmers use the conventional methods such as spray of fungicides and bactericides for the control of these infections. But these agrochemicals are often expensive and have residual toxicity. The fungal pathogens namely, *Alternaria* sp., *Aspergillus* sp., *Fusarium* sp., *Penicillium* sp. and *Phomopsis* sp. have been described in the cause of pomegranate fruit rot [2]. *Alternaria alternata*, *Coniella granati* and *Phytophthora* sp. have also been reported to cause fruit rot of pomegranate [11]. *C. raphigera* is a known phytopathogen. It is reported

Fig. 3 Extracellular enzyme activity of *Chaetomella raphigera* for pectinase (a), xylanase (b), cellulase (c) and protease (d) (Left side). Pectinase (filled diamond), xylanase (filled square) and cellulase

(filled triangle) activity in tissues (fruit) artificially infected with *C. raphigera* and its correlation (right) with disease rating scale (triangle)

from India to cause leaf spot disease of *Cuphea* sp., an oilseed crop [12]. *Chaetomella raphigera* is also pathogenic to blueberry (*Vaccinium corymbosa* L.) as reported in Florida [5]. *C. raphigera* is described here for the first time as a pathogen of pomegranate fruit rot from India. The fungal pathogen *C. raphigera* was responsible for mild form of rot of pomegranate (Fig. 1e). The rotting was present up to a depth of 5 mm and the rotting diameter on the fruits reached only to 15–16 mm corresponding to the disease rating scale of 3.

Although disease severity caused by *C. raphigera* is less, it is still of interest as it decreases the market value of fruits. The present investigation is the first report of morphological and molecular identification of *C. raphigera* as a pomegranate fruit rot pathogen. Understanding, which enzymes play important role in the virulence of fungal pathogens, is necessary to develop formulations with enzyme inhibitors thereby minimizing injury to the fruits.

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