

Phytotoxicity Studies of *Ceratocystis fimbriata* Causing Pomegranate Wilt in *Punica granatum* L. Cv. Kandhari Kabuli

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Abstract

Ceratocystis fimbriata causing wilt disease in pomegranate was isolated and purified on potato dextrose agar medium from infected roots of *Punica granatum* L. cv. Kandhari Kabuli. Microscopic examination of a fifteen days old culture revealed septate conidiophores and hyaline conidia (10 to 15 µm long) and perithecia were black with a globose base (100 to 300 µm). Ascospores exuded from the apex of the perithecium neck in a long coil and were small, hyaline, and hat-shaped. The isolated culture was inoculated to one year old plantlets of pomegranate and also incubated with healthy leaves in a growth chamber. Typical symptoms of wilting were observed in both inoculated seedlings and detached leaves. The isolated culture filtrate also confirmed the presence of toxic metabolite in it as the *in vitro* callus inoculated in pure culture filtrate showed browning and death of the cells. Thus, an efficient method was developed for the identification of pathogen and extraction of culture filtrate from it which can be used as a selection agent for developing disease resistant plants.

Keywords: *Ceratocystis fimbriata*; Pomegranate wilt; Culture filtrate; Phytotoxicity

Introduction

Pomegranate wilt disease caused by *Ceratocystis fimbriata* is one of the important diseases of pomegranate adversely affecting crop cultivation in all major growing regions of India. At present, the crop is severely affected by wilt pathogen and day by day, the wilting severity is increasing at faster rate. It was first noticed in two areas of the Bijapur district of Karnataka, India in 1990 which rapidly spreaded in the entire Bijapur district. The cause was not identified until 1995, however, the fungus *C. fimbriata* was isolated from discoloured stem, root, and branch tissues on wilted plants in 1996. The disease is prevalent in parts of a Maharashtra, Karnataka, Andhra Pradesh, Gujarat and Tamil Nadu states [1-2] in India.

Pomegranate wilt results in complete wilting of plant and is characterized by the initial symptoms as yellowing and wilting of leaves on one to several branches. Initially symptoms only occurred on shoots, but later, leaves of the whole tree turned yellow and wilted, causing extensive defoliation and dieback and the xylem of the trunk turned brown to black with a star burst-like pattern. Finally, heavy infection resulting in the whole tree dying, causing severe yield losses leading to death of affected plants in a few weeks [1]. Fungi known to produce biologically active secondary metabolites in their cultures filtrates, often displaying phytotoxicity in tests on tissue culture plantlets, calli and cellular suspensions of many plant species. Experiments with pathogen culture filtrates have shown that tissue response *in vitro* may correlate with disease reaction of the host variety and, where this occurs, the use of culture filtrates may allow selection of important traits in disease resistance *in vitro* [3]. It is well known that inoculation with culture filtrates of some plant pathogenic fungi can produce disease like symptoms and may also be used to select for resistance [4-7]. Culture filtrates are mostly produced by fungal cultivation in liquid media and subsequent separation of the solid and liquid parts of the culture. The liquid part of the culture is used as the selection agent. Crude culture filtrates have been used as selective agents in numerous disease resistance studies in which they exhibit phytotoxic activity [7,8].

Thus in the present study an attempt was made to characterize pathogenic behaviour of *Ceratocystis fimbriata* and also to prove the phytotoxicity of its culture filtrates on *Punica granatum*.

Materials and Methods

Collection of diseased samples

The roots and soil samples of pomegranate plants severely infected by *Ceratocystis fimbriata* were collected from Regional Research Station, Bajaura of Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan, HP, India.

Isolation, purification and morphological studies of *C. fimbriata*

The disease sample was first washed with tap water and small bits of size 0.5 – 1.0 cm were cut with the help of sterilized blade. The bits were surface sterilized by dipping them for 30 seconds in 0.2% bavistin and 3 minutes in 0.5% sodium hypochlorite and washed thrice with sterile distilled water. The bits were then dried on pre sterilized filter paper to remove the excess of moisture and were aseptically transferred on to potato dextrose agar plates. The inoculated plates were incubated at 25 ± 1°C and frequently examined for mycelial growth. The culture of pathogen *C. fimbriata* was purified by standard hyphal tip isolation procedures and then purified culture was maintained on potato dextrose agar slants and kept in a refrigerator at 5°C, for further use. In order to confirm the identity of the fungus, the ascospores and perithecia were observed under microscope.

Ex vitro inoculation of pathogen for testing the pathogenicity

To test the pathogenicity fresh suspension of pathogen is required. The pathogen was grown on PDA for four to six days at 25 ± 1°C. After 48h of incubation at 25 ± 1°C the mycelia mat obtained was harvested and homogenized in sterile distilled water. The mixture was

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strained through muslin cloth and the suspension thus obtained was immediately used for pathogenicity test. For testing the pathogenicity, 10 ml of freshly prepared mycelia suspension was sprayed on one year old plants of *Punica granatum*. Optimum humidity was maintained by irrigating the pots. These seedlings and leaves were then observed regularly for initiation of symptoms.

In vitro inoculation of pathogen for testing the pathogenicity

In detached leaf inoculation technique three middle aged leaves were selected and detached from the plants. They were washed well in tap water, swabbed with 70% ethanol and allowed to dry. Then injuries were made at several points by pricking with sterilized needle charged with inoculum and smeared on both sides with culture soaked sterilized cotton swab. The leaves were kept in plates which were lined with sterilized moist filter paper to maintain humidity and incubated at 25°C.

PCR amplification of ITS region

The two internal transcribed spacer regions (ITS1 and ITS2) and the 5.8S gene of the ribosomal RNA operon were amplified using primers ITS1 and ITS4 (White et al., 1990). Polymerase chain reaction (PCR) mixtures consisted of 1X PCR buffer, 0.2 µl of 10 nM each primer, 0.6 µl of 0.5 mM each dNTP, 1U taq DNA polymerase, 20–30 ng DNA. Reaction volumes was adjusted to 20 µl with autoclaved distilled water. The PCR program was set at 95°C for 15 minutes, followed by 35 cycles at 94°C for 1 minute, 54°C for 2 minutes and 72°C for 2 minutes and final amplification at 72°C for 10 minutes.

Sequencing and BLASTn analysis

The PCR product obtained through amplification with universal primers were sent for sequencing using same upstream and downstream primers to Xcleris lab, Ahmedabad, India. ITS region sequences of isolated fungus was analyzed using BLASTn to align them with corresponding sequences of ITS from the database.

Extraction of culture filtrate

The pure culture of *Ceratocystis fimbriata* was cultured in Liquid Potato Dextrose medium and incubated at 25°C for 30 days. The fungal culture in liquid medium was filtered by 3 layers of sterilized muslin cloth, followed by previously sterilized ordinary filter paper. Culture filtrate was collected by gravity through Whatman No. 1 filter paper. Culture filtrate was then subjected to centrifugation at 10,000 rpm for 20 minutes to collect supernatant. Supernatant was sterilized by passing it through a 0.22 µm nitrocellulose. After filter sterilization, the culture filtrate was incubated for 7 days at 25°C to check the growth of fungus if there were any spores left after the filtration.

In vitro testing of toxicity of the culture filtrate

Various culture conditions such as time of culture, temperature and composition of the medium were some of the factors, which may change the pathogen from virulent to avirulent form. Therefore, it became necessary to assess the toxicity of culture filtrate. For this, small pieces of *Punica granatum* callus were incubated in 100 per cent culture filtrate of *Ceratocystis fimbriata*. For comparison, autoclaved distilled water and potato dextrose broth (medium, which was used to grow the fungal culture as it had the same composition as the culture filtrate except for the toxin) were used as control. Both control and treated callus were incubated in culture room and observation regarding effect of toxin were recorded daily.

Results

Isolation, multiplication and maintenance of pure culture of fungus (*Ceratocystis fimbriata*)

C. fimbriata was isolated from the infected roots of trees showing typical symptom of pomegranate wilt. After inoculation culture was incubated at 25 ± 1°C for one week till uniform growth was obtained. The isolated fungus (*C. fimbriata*) grew well on Potato Dextrose Agar and produced whitish grey mycelium which changed to brown colour with age owing to production of micro, macro conidia and perithecium. Black coloured perithecia with a globose base were observed (100 to 300 µm), exuding small, hyaline, and hat – shaped ascospores from the apex of the perithecium neck in a long coil (3.0 to 5.0 µm long × 2.0 to 4.0 µm wide). Conidiophores were septate with hyaline conidia, 10 to 15 µm long × 6 to 15 µm wide (Figures 1a-1d).

Testing the pathogenicity of fungus

During *ex vitro* testing symptoms of wilting started appearing such as yellowing of the leaves in plants sprayed with fungal suspension (Figure 1e (ii)) after 15 days of inoculation whereas in case of control no such symptoms were observed (Figure 1e (i)) However, in *in vitro* testing by in detached leaf technique yellowing followed by necrosis of the leaf was observed within eight to ten days after inoculation.

Characterization of isolated fungus using universal primers

The isolated fungus was further characterized using ITS gene technology and after 35 cycles of PCR amplification universal primers were able to successfully amplify the entire ITS region and produced an amplicon of size 346 bp. Further *in silico* analysis pertaining to the sequences, so obtained, was carried out using various BLASTn analysis available online. Analysis of ITS revealed its homology with various other ITS gene sequences. Characterization of the fungal isolate on basis of the ITS gene coding genes revealed that it showed maximum similarity with *Ceratocystis* species. Resemblance of obtained ITS sequences with the analogues available in database of computer program “BLAST” is presented in Table 1.

Testing toxicity of culture filtrate

To test the toxicity of culture filtrate small pieces of *Punica* callus

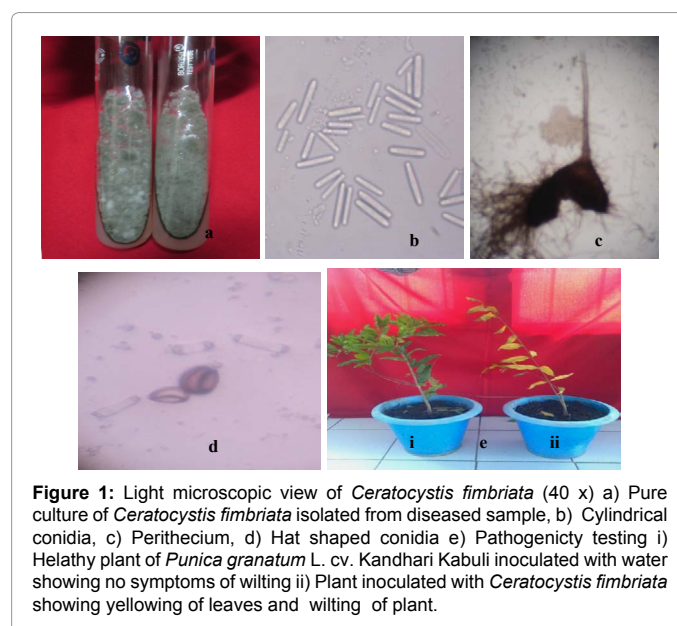


Figure 1: Light microscopic view of *Ceratocystis fimbriata* (40 x) a) Pure culture of *Ceratocystis fimbriata* isolated from diseased sample, b) Cylindrical conidia, c) Perithecium, d) Hat shaped conidia e) Pathogenicity testing i) Healthy plant of *Punica granatum* L. cv. Kandhari Kabuli inoculated with water showing no symptoms of wilting ii) Plant inoculated with *Ceratocystis fimbriata* showing yellowing of leaves and wilting of plant.

were incubated in 100 per cent culture filtrate of *Ceratocystis fimbriata* for 48 hours. After 48 hours, small pieces of callus which were incubated in 100% culture filtrate turned brown (Figures 2a and 2b) and after 7 days of incubation the callus was dead, whereas in case of control no such browning was observed (Figures 2c and 2d). This confirms the toxicity of culture filtrate that had caused the death of the cells.

Discussion

Fungal culture filtrates contain a spectrum of secondary metabolites, such as polysaccharides, oligosaccharides [9], proteins, glycoproteins, unsaturated fatty acids, along with toxins (host-selective and non-host-selective) that may play a role as co-determinants of pathogenicity during disease development [6,7,10,11]. The application of culture filtrates to cultures *in vitro* can trigger the elicitation of various defense responses, e.g., phytoalexins; activity of certain enzymes, accumulation of phenolic acids [12], total phenols, peroxidases and beta 1, 3-glucanase and chitinase [13]. It is well known that inoculation with culture filtrates of some plant pathogenic fungi can produce disease-like symptoms and may also be used to select for resistance [4,5,7]. Crude culture filtrates allow easy bioassay and screening for toxic effects on plants, cuttings, leaf discs or even cell suspension cultures of the host species [14]. In the present investigations, *C. fimbriata* was found to release toxic metabolite into the medium that was isolated from culture filtrate. Callus tissues are more sensitive than intact plants. Thus the tissue culture technique provides a good experimental tool for precise. evaluation of the phytotoxicity of fungal toxic metabolites *in vitro* [15]. The callus tissue undergoes necrosis and brownish

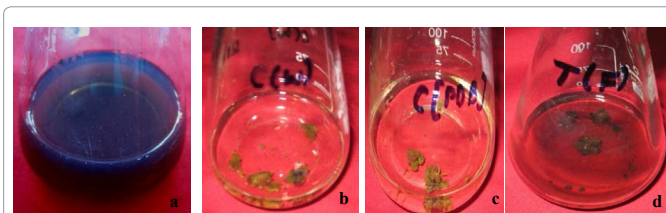


Figure 2: a) Pure culture filtrate of fungus *Ceratocystis fimbriata* after 30 days of incubation, b) Callus incubated in water (control) after 1 week, c) Callus incubated in potato dextrose broth (control) after 1 week, d) Callus incubated in fungal culture filtrate showing browning and release of phenolics due to toxic effect of filtrate.

discoloration because of the accumulation of phenolic compounds and their products [16]. Similar browning of callus tissues was observed in the present studies. A progressive decrease in the callus growth observed with the increasing concentration of toxin is in conformity with the previous reports of many other plant-callus and pathogen-toxin interactions [16-18]. Results of the present studies revealed the ability of *C. fimbriata* to produce phytotoxic compound in the culture filtrate. Thus, the involvement of this toxin in the development of wilt disease symptoms is a possibility. This study provide insights regarding evaluation of somaclonal variation for disease resistance in *Punica granatum*, with respect to availability of an efficient and reliable screening method wherein the effect of the selection agent (pathogen culture, culture filtrate) can be evaluated over different concentration ranges.

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S.No	Closest Match	Percent Similarity	Accession Number
1	<i>Ceratocystis fimbriata</i> strain Ayy316 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	99%	HQ529711.1
2	<i>Ceratocystis fimbriata</i> isolate C2055 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence	98%	HQ157548.1
3	<i>Ceratocystis fimbriata</i> isolate C1889 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence	98%	HQ157547.1
4	<i>Ceratocystis fimbriata</i> isolate PL01 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	98%	KJ469356.1
5	<i>Ceratocystis fimbriata</i> isolate PC03 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	98%	KJ469347.1
6	<i>Ceratocystis fimbriata</i> isolate PB67 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	98%	KJ469346.1
7	<i>Ceratocystis fimbriata</i> isolate C2055 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence	98%	HQ157548.1
8	<i>Ceratocystis fimbriata</i> isolate C1889 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence	98%	HQ157547.1
9	<i>Ceratocystis fimbriata</i> isolate C2041 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	98%	AY585345.1

Table 1: Similarity values of ITS sequence of selected fungal isolate.

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