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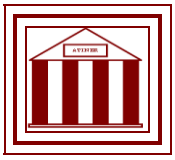

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Sensitive Detection of *Aspergillus tubingensis* Causing Heart Rot in Pomegranate (*Punica grantum* L.) and its Biocontrol by Indigenous Phyto-extracts

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Abstract

Pomegranate (*Punica grantum* L.) is an edible fruit of great nutritional, medicinal and economical importance. In a field survey, heart rot disease was observed on Pomegranate in different areas of Pakistan including Islamabad and Murree. Typical heart rot symptoms like dark brown to black spots in the form of wrinkles and lesions were observed in different orchards. Affected fruits were collected for the isolation and identification of disease causing pathogen. This study was also extended to see antifungal effect of selective medicinal plant extracts to suppress and control this disease. On the basis of mycelium morphology and sequence analysis, *Aspergillus tubingensis* was found to be the disease causing pathogen. Blast analysis revealed 100% similarity with 18S rDNA, partial sequence; ITS-1, 5.8S rDNA, and ITS-4, complete sequence; and 28S rDNA gene, partial sequence (Accession no. HM037959.1). This is the first ever report of *A. tubingensis* causing heart rot of pomegranate in Pakistan. Extracts of *Sygygium aromaticum* and *Cinnomomum verum* showed maximum mycelial growth inhibition while *Euphorbia granulata* exhibited the least. These results suggest an effective and environment friendly control of *A. tubingensis* on Pomegranate.

Keywords: *Aspergillus tubingensis*, Heart rot, Pomegranate, *Sygygium aromaticum*, 18S ribosomal RNA gene.

Introduction

Pomegranate is native to central Asia and it is a symbol of abundance and prosperity (Bhowmik et al., 2013). Pomegranate is a non-climacteric fruit that only ripens on the tree.Fruit can be picked or collected from the tree after its complete ripening. Pomegranate is a deciduous trees, and it has been reported to be originatedfrom Iran and Himalayas.This tree is now cultivated in the Mediterraneanbasin, North and South Americaand also in Central Asia (Chandra et al., 2010). Pomegranate is afavourite fruit of tropical and subtropical regions of Pakistan. It is cultivated on 13283 ha with a production of 50109 tons in Pakistan (Anonymous, 2006). Pomegranate is grown in Mediterranean environments with cool winters and hot summers and injured atleass than -11°C temperatures (Morton, 1987).

The pomegranate is about 12-16 feet in height with multiple branches. Different parts of pomegranate tree like seed, peel, leaf, bloom and root bark are widely utilized in therapeutic and food materials, due in great health benefits for atherosclerosis, cholesterol levels and cancer prevention (Akpinar-Bayizit et al., 2010). Pomegranate fruit has great nutritional value. People use pomegranates as fresh fruit and its juices are also considered very delicious. The arils of Pomegranate contain polyphenols, which show high antioxidant activity. It is also beleived that the pomegranate prevents cardiovascular diseases (Gil et al., 2000). In Ayurvedic medicine,it is utilized as a “blood tonic,” and it heals. Pomegranates provides antioxidants to fight with radicals.

Many reports reveal that Pomegranate fruit rots is one of the most yield limiting factors. Many abiotic factors including chilling stress and husk scald cause its weight loss (Selcuk and Erkan, 2014). Pomegranate is an improtant fruit but the information about diesease causing pathogens of its fruits is still scarce. Many pathogens including *Alternaria spp*. and *Aspergillus spp*. have been reported to cause infections of pomegranate fruit.

Heart rot is a tyical disease in which fruit core becomes black and disease inititates from calyx area. In this disease, hard rind and peel retain their healthy look.Advanced infection may result in fruit that is less dense with a mottled and puffy rind that often has darkened coloration. Many infections do not display any external cues about the extent of infection. The typical extent of infection is over 50% of the volume, but may also be limited to a discolored lesion size of a dime (Ezra et al., 2015).

During 2014-2015, massive Heart rot of Pomegranate was observed in several fields of Islamabad and Murree. Pakistan. The disease incidence was observed to be more than 30% and symptoms of heart rot included dark brown rotting on fruit. As the time progressed, fruits were severely damaged and fall off the plant. The objective of this study was to identify the disease causing pathogen.

Materials and Methods

Collection of Samples

Thirty five diseased samples with typical heart rot symptoms were brought to the laboratory from different areas of Murree and Islamabad, Pakistan. Most of the disease severity was observed in Islamabad (30 % of randomly selected 35 trees), followed by Rawalpindi (28 % of randomly selected 40 trees) and Murree (26 % of randomly selected 35 trees). Out of these 35 diseased samples, few were less affected and most were severely rotten. The infected samples were collected in disinfected polythene bags, labelled and brought to Plant Pathology Laboratory, Department of Plant Sciences, Quaid-i-Azam University, Islamabad for the isolation and recognition of causal agent and analysis of pomegranate disease.

Isolation of Fungus Pathogen

Affected pomegranate fruits were cut into two parts with a sterilized knife and surface sterilized by putting in 1% Sodium hypochlorite solution for three minutes and washed thrice with distilled water. The pieces of infected fruits were placed on autoclaved moistened filter papers in sterilized Petri plates and put in an incubator for 3 days at 26-28 °C for isolation.

Fungus Growth on Media

To grow fungus, oxy-tertacycline (OTC) was added in PDA media (1ml/100ml), as an antibacterial factor. Fungus was aseptically transferred to the media of petri plates with the help of sterilized spatula and placed at 25±3 °C for 6-7 days. Fungus cultures and the morphology of conidia was observed from 1–3 week old cultures on PDA. Light microscope and stereoscope were used to see the shape, size, and length and width of conidia and acervuli.These results confirmed fungus morphology.

Pathogenecity Analysis

To confirm the pathogenecity of isolated fungus, Koch's postulates were followed and the isolated fungus was used to inoculate healthy fruits by the following two methods.

Non-invasive Wound Inoculation

Wounding method was used to inoculate fungus on healthy fruits of pomegranate. For this purpose, wounding of 25 fruits of *pomegranate* was performed with the help of sterilized spatula. Five wounded fruits were kept as control while 5mm plug of mycelium was taken from fresh culture of fungus and placed on twenty remaining fruits. All fruits were covered with sterilized muslin cloth to save them from other pathogens attack. Three days post inoculation, disease symptoms were observed on daily basis in those

inoculated fruits. The affected fruit parts with typical symptoms of blakish brown lesions on skin were used for the re-isolation of pathogen on PDA. The structure of pathogen wasobserved again for itscomparison with initially isolated pathogen. This experiment was repeated thrice, following the directions of Chai et al. (2014).

Foliar Inoculation

Foliar inoculation was performed according to standard protocol (Ibrahim et al., 2014). Twenty pomegranate fruits were sprayed with a conidial suspension (10^6 conidia mL^{-1}) and ten were sprayed with sterilized water, which served as control. Symptoms appeared one week post foliar inoculation. Casual pathogen was re-isolated on PDA from the fruits with distinctive brown spot symptoms. The structure of the pathogen was confirmed.

Recognition of Fungal Isolates

The tiny piece of mycelium, from the colony of fungus was placed along the slide onto which the drop of stain was placed with the assistance of a mounting needle. This needle was also employed to spread the mycelium on the coast. The slide was observed at 40X and 100X magnification. The pictures were captured from a light microscope camera and the species were identified following the instructions of Cheesbrough (2000).

Molecular Identification of Fungus

The fungus isolates were identified by the sequence analysis of (18S-ITS1-5.8S-ITS2-28S). For sequencing, fungus was grown on PDA media and its DNA was extracted by standard CTAB method (Lee and Taylor, 1990). The amount and quality of extracted DNA were determined by spectrophotometer. The forward primer was designed from 18S rDNA (5′ CCTTGGGATCGGCATTGATG3′) and reverse primer was designed from 28S rDNA (5′ GATGCTCACACACTGCGATC3′) to amplify specific rDNa region including 18SrDNA-ITS1-ITS2-28SrDNA (White *et al*., 1990). For PCR,a reaction mixture (50 µL) was prepared using dNTP (6 µL), *Taq* polymeraseenzyme (1.5 µL), primers (1 µL each), fungal DNA (1 µL) and 10 × polymerase buffer(5 µL). The gene amplification by PCR was performed at 95 °C for 5 minute followed by 32 rounds of 94 °C for 50seconds, 58 °C for 50seconds, 72 °C for 50secondsand a final extension period of 72 °C for 8 minute. The resultant product was sequenced and explored (http:// www.ncbi.nlm.nih.gov). By using clustal W, the sequences of similar and related species of *A. tubingensis*, available on GenBank, were used for alignment. This aligned data was used to construct the neighbor joining tree, using MEGA6 with 1000 bootstrap value (Tamura et al., 2007).

Use of Phyto-Extracts to Control Heart Rot Disease

Extracts of *Momordica charantia, Ricinus comminus,Sygygium aromaticum*, *Cinnomomum verum*, and *Euphorbia granulata*were used *in vitro* and *in vivo,* to see their effect on fungal growth inhibition.

Preparation of Plant Extracts

Leaves of above mentioned medicinal plants were surface sterilized with 70% ethanol, rinsed twice with sterilized water and placed is shade to dry. These dried leaves were used for grinding to powder form and plant material (2 g) was suspended in sterile distilled water (20 ml) to make its 10 % (w/v) concentration. These suspensions were briefly stirred until boiling and cooled for 5 minutes. Through sterile cheesecloth, suspensions were filtered and the extracts were autoclaved and stored at 4 °C.

Spores and Conidial Growth Inhibition of *A. tubingensis* (*In Vitro*)

Poisoned food technique was used for *in vitro* anti-fungal activity evaluation of aqueous plant extracts (Nene and Thapliyal, 1979). For this purpose, 5ml of each plant extract was poured into 15-18 ml of autoclaved Potato dextrose agar (PDA) medium, amended with streptomycin, and carefully agitated for thorough mixing of extract and media. The amended media (15 ml) were dispensed into 90 mm Petri dishes. *A. tubingensis* was grown on PDA and 5 mm disc of this fungus was was placed in the centre of all plates and incubated at 26±1°C for 6-7 days. Medium amended with sterilized water, served as control. Each treatment was replicated thrice. Six days post incubation, the diameter of the fungus colony was measured from each treatment to calculate mycelial growth inhibition.

Disease Control Assay (*In Vivo*)

Above mentioned plant extracts were used for the control of black heart disease on pomegranate, *in vivo,* following a standard protocol (Itako et al., 2008). Pomegranate fruits were inoculated with *A. tubingensis* afterwounding. One day later, these inoculated fruits were sprayed with plant extracts and distilled water was sprayed as a control. Two weeks post inoculation, disease severity was calculated by using scale method (Téliz-Ortiz et al., 2003).

Results

Symptoms of Pomegranate Heart Rot

The symptoms of heart rot were dark brown in the form of lesions that started near calyx on fruit (Figure 1 A-C). With the passage of time, severity of disease increased. Surface of fruits was apparently less affected but internally, they were more rotten. As disease progressed, fruits were severely damaged and fall off the plant.

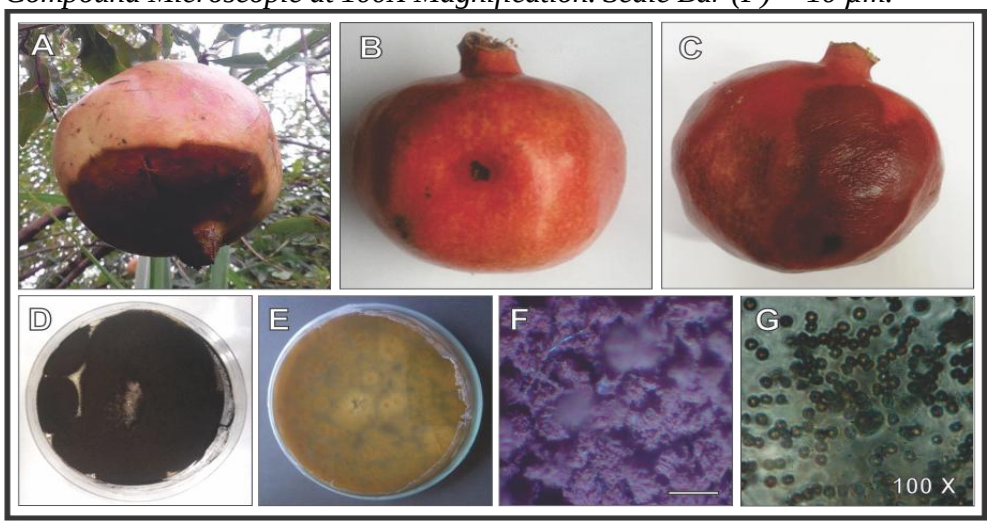
Isolation and Identification of Fungus

Disease affected fruit parts were placed on PDA media and twenty two identical isolates were found from thirty fruit.From plated tissues, recovery requency of isolated was 83%. The causal fungus morphology was observed in Petri plates. Initially, white mycelium appeared that later changed to dark black with a net-like structure. The colony on the plate from reverse side was yellowish and morphologically, it was similar to *Aspergillus tubingensis* (Figure 1G).

Pathogenicity Test

After wounding and foliar inoculation, typical brown spots appeared on fruits. After wounding inoculation, symptoms of heart rot were less pronounced and appeared slowly in 1st week and progressed further in 2nd week (Figure 1D-F). Initially, small spots were observed which ranged from 2-4 mm and later enlarged to 2-4 cm. Control plants showed no symptoms, even after 3 weeks of inoculation. To confirm the disease causing agent from these self-inoculated fruits, fungus was re-isolated, on the same media. Re-isolated fungus was again observed to be *A. tubingensis*. In accordance with Koch's postulates, these results confirmed the involvement of *A. tubingensis* in heart rot disease.

Figure 1. (A) *Typical Heart Rot Symptoms were Observed on Pomegranate* (B) *Disease Causing Pathogen was Isolated on SDA and Inoculated on Fresh Fruits* (C) *This Infection Produced Similar Symptoms* (D) *Fungus Morphology was Observed on PDA Media from Front Side* (E) *and Back Side* (F) *For Further Confirmation, Fungus was Observed under Steroscope* (G) *and Compound Microscopic at 100X Magnification. Scale Bar (F) = 10 µm.*



Molecular Identification of Isolated Fungus

The fungus was identified on the basis of molecular analysis by sequencing ITS1, ITS4, 5.8S, 18S, and 28S rDNA homology. Sequence alignment was carried out and BLAST analysis revealed 100% similarity with

A. tubingensis strain, 18S rDNA, partial sequence; ITS1, 5.8S rDNA, and ITS4, complete sequence; and 28S rDNA, partial sequence (Accession no. HM037959.1). These results confirmed our previous observation based on visual and microscopic morphology of fungus.

Microscopic Study of Fungus

Guttulate hyphae, uniserate conidiophores and phialides lecythiform were visualized at 100 × magnification (Figure 1G). Mycelium was white cottony which became black as conidia developed. Conidia were pale brown to reddish brown in mass, globose to subglobose, tuber culateechinulate. Colonies formed were black with asexual spores of fungus. On the basis of these images, this fungal species was identified as *Aspergillus tubingensis.*

Effects of Plant Extracts on the Growth of Aspergillus tubingensis

Plant extracts have environment friendly nature therefore; these are preferred over chemical fungicides in the modern era. Both *in vitro* and *in vivo* disease resistance evaluation described the significant role of aqueous plant extracts to suppress the growth of *A. tubingensis* (Figure 2, Table 1). Extracts of *Syzgium aromaticum*and *Cinnamomum verum* exhibited strong antifungal activities against *A. Tubingensis, in vitro*as well as *in vivo* (Figure 2, Table 1). *Memordica chrantia* also showed remarkable inhibition of this fungus (Figure 2). *Ricinnus comminus* and *Euphorbiagranulata* showed least fungal inhibition properties, as compared to others. These results suggest *in vitro* and *in vivo* use of *Syzgium aromaticum* and *Cinnomum verum* for the control of *Aspergillus tubingensis* in pomegranate.

Figure 2. *Antifungal Activity of Different Extracts against A. tubingensis (In Vivo)*

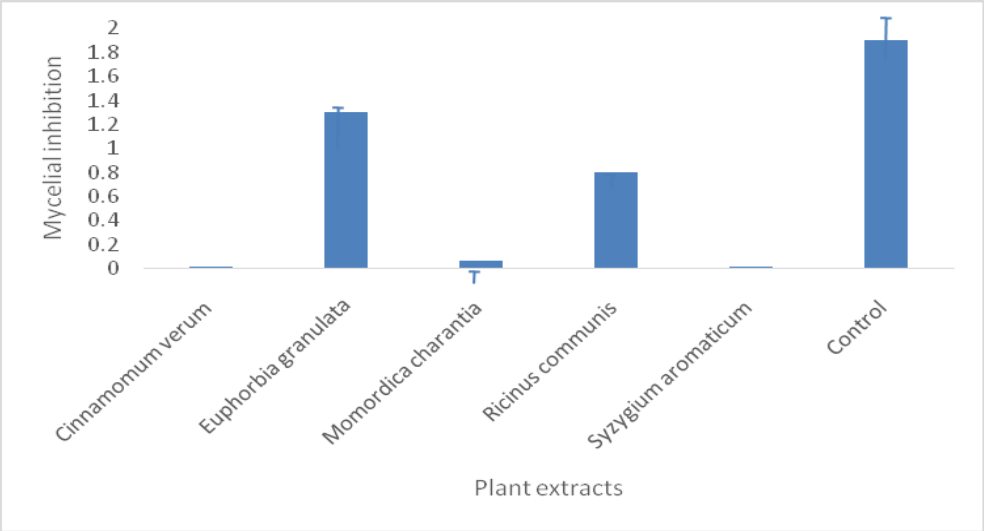


Table 1. *In Vitro Antifungal Activity of Different Medicinal Phyto-Extracts for the Control of A. tubingensis*

Name of plant extract	**Diameter(cm)**
Cinnamomum verum	0.02
Euphorbia granulate	1.3
Momordica charantia	0.07
Ricinus communis	0.8
Syzygium aromaticum	0.02
Control	1.9

Discussion

Various fungal pathogens such as *Alternaria* spp., *Botrytis cinerea*, *Aspergillus niger* and other *Aspergillus spp*., *Colletotrichum gloeosporioides, Coniella spp*., *Nematospora spp*., *Pilidiella granati*, *Penicillium spp*. and *Rhizopus spp.*cause fruit decay (Palou et al., 2013). This study depicts the precise recognition of the pathogen causing heart rot of pomegranate fruit in different areas of Pakistan. Species in the genus *Aspergillus* also cause similar diseases in Cyprus and Greece (Martinez-Culebras and Ramon, 2007). *Aspergillus* species also cause food spoilage and produce mycotoxins among which aflatoxins and ochratoxin A are the most important (Pitt and Hocking, 1997). The presence of this fungus pathogen on fruits could pose a serious threat to the health of its consumer. The presence and subsequent spoilage due to *A. tubingensis* could become a serious cause of health hazards and possible economic loss. Uses of common fungicides which are available in market for the control of pathogens are injurious to human health and also the cause of environmental pollution. Therefore, the use of biofungicides is preferred for the control of fungus to protect plant and human health. Plant metabolites and plant based pesticides are better alternatives with minimal environmental impact (Verma and Dubey, 1999).

In our study, antifungal effects of *Syzygium aromaticum* and *Cinnomomoum verum* were very prominent and promising. Clove (*Syzygium aromaticum* L. Merrill and Perry) is one of the commonly used spices in Pakistan. It is used as a traditional medicine and food preservative (Cortés-Rojas et al., 2014). Its flower budswork as antiviral, antimicrobial and antifungal general stimulant (Politeo et al., 2010). *Momordica Charantia* and *Datura Alba* also showed good results for fungal control. The anti-diabetic properties of bitter gourd have been well documented (Basch et al., 2003). All medicinal plants do not exhibit similar anti-microbial activity. The difference in the activity of plants might be due to the differences of chemical compounds and their different amounts in each plant. This is the 1st report of *Aspergillus tubingensis* causing heart rot of pomegranate in Pakistan.

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