Pak. J. Biochem. Mol. Biol. 2015; 48(2): 57-60

Genetic characterization of *Scorias spongiosa* identified in citrus: a first report from Pakistan

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Abstract: *Scorias spongiosa* (Schwein.) Fri. is a fungal pathogen that grows and invades leaf epidermis and consequently retards photosynthesis. During infection, it utilizes sugar exudates of aphids. The study aims at morphological and molecular characterization of this pathogen on citrus. The *Citrus sinensis* exhibiting any sign and symptom of fungal infection were selected and leaf samples were collected from Nowshera, Pakistan. Microscopic analysis of infected citrus leaves revealed thick mycelia on the upper epidermis covering stomata. Total RNA was extracted from mycelia taken from fungal colonies on infected citrus leaves using NucleoSpin plant RNA isolation kit and reverse transcribed, accordingly. Nucleotide alignment (BlastN) of 18S rRNA gene amplified with universal primers exhibited 96 % homology with this fungus. The phylogenetic lineage of the amplicon with available databases suggests this strain as exotic which migrated from China to Pakistan. This is the first report of the presence of *Scorias spongiosa* in Pakistan.

Keywords: Capnodiaceae, phylogenetic analysis, sooty molds, 18S rRNA**.**
Received: August 29, 2014 **Accepted:** February 20, 2015
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INTRODUCTION

Citrus (Rutaceae) is an important economic crop grown in the tropics and subtropics of the globe. It harbors valuable natural products including vitamins and minerals, which determine its nutritive potentials [1]. Pakistan is one of the top citrus producing nations and the area under cultivation is ~198.4 thousand hectares with an annual production of 2,150 thousand tones [2]. Citrus is susceptible to a number of pathogenic diseases resulting in serious economic deficit. Being dominant, the fungal pathogens cause postharvest diseases on its fruits altering horticultural parameters [3]. However, the mode of systemic infection varies with different fungal species; some directly infect the citrus, whereas other such as sooty molds invades by inhabiting on the sugary exudate secreted by insects.

Sooty molds refer to a group of species which characteristically belong to families Antennulariellaceae, Chaetothyriaceae, Capnodiaceae, and Metacapnodiaceae [4,5]. They typically grow on leaves as a black growth that deters photosynthesis by reducing sunlight and results in low yield of fruit [4]. The complexes of sooty mold comprising common genera of fungi are *Cladosporium, Aureobasidium, Fumago, Antennariella, Limacinula, Scorias, and Capnodium.*

Scorias (Capnodieceae) was named after *Scorias spongiosa* (Schwein.), used to signify the structure formed on leaves and branches of the *Fagus grandifolia* L. [7,8]. *Scorias spongiosa* lives as saprobe on sugar exudate of aphids. This pathogenic fungus is known to be distributed along the U.S East coast from Northern Florida to Maine[8]. Recently in Kenya, *Scorias spongiosa* was identified on the sugary exudate of Coccus species on several unrelated plants, including *Coffea arabica*, *Citrus sinensis*, *Eriobotrya japonica* and *Ehretia cyimosa* [9]. In early studies, it was described that the hyphae of the fungus as copious, syncytium and somewhat slimy while the pseudothecium centrum is characterized as cartilaginous-gelatinous due to the nature of component sterile elements [10]. However, the dense black thallus is usually 3.8-5.5 µm in size and consists of septate mycelia. An ascomata may encompass several asci each of which contains eight bitunicate spores [11]. Moreover, fungal thallus was observed to be a cream to buff colored tufts of mycelium which develops during fall to winter season. The detailed developmental morphology of *Scorias spongiosa* was also described [8]. Besides morphological characterization, genetic analysis keeps some importance for identification and diagnosis of fungus. In addition to traditional methods, amplification of specific genomic region via polymerase chain reaction can be used for quick and reliable identification of fungal species within a community [12]. Amplicon sequencing of fungal genome (ribosomal genes) has appeared as a useful method for the identification and detection of pathogenic fungus [13]. Traditional methods of pathogenic fungal identification are ambiguous and may lead to misidentification. However, molecular techniques can provide accurate information about specie identification by comparing DNA sequence information among known and unknown fungal species. The main purpose of this study was to determine the efficacy

of using RT-PCR to amplify the 18S rRNA region of fungal genome (*Scorias spongiosa*) for the purposes of sequencing and subsequent species identification.

Herein we have reported the morphological identification and genetic characterization of *Scorias spongiosa* on citrus for the first time in Pakistan. As citrus plants have been found to be affected by several fungal diseases and many of them have been identified in Pakistan.

MATERIALS AND METHODS

Sample collection

Infected leaf samples of *Citrus sinensis* exhibiting black growth on the upper side were collected from Nowshera (33°59'48N 72°0'47E), a district of Pakistan in the province Khyber Pakhtunkhwa, which accounts for around eight percent of citrus fruit production in the province.

Microscopic analysis

Leaf samples were prepared according to the modified method [14] and were examined using microscope (NIKON eclipse 80i) equipped with digital camera.

RNA extraction, cDNA synthesis and PCR

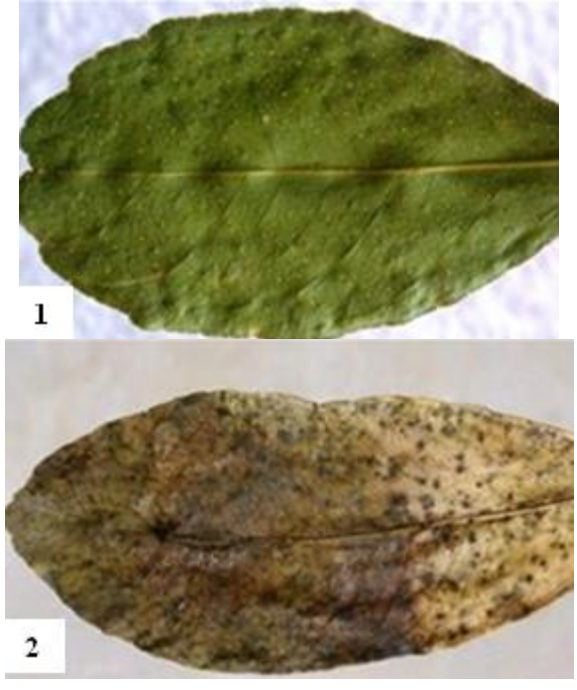
Total plant RNA was isolated from 100mg of homogenized leaf tissue using NucleoSpin plant RNA isolation kit according to the manufacturer's recommendations (Macherey-Nagel, Düren, Germany). Optical Density (OD) of extracted RNA was checked by spectrophotometer at 260-280 nm[15]. Complementary DNA (cDNA) synthesis was performed using 1µl of Moloney murine leukemia virus (MMLV) reverse transcriptase enzyme (Fermentas, USA) starting with 10 µl of total RNA in a reaction mixture of 20 µl with universal anti-sense primer. Reverse transcription was run for 55 minutes at 37ºC and then for 3 minutes at 95ºC on MultiGene Thermal cycler (Labnet). The 18S rRNA gene was amplified from the resulting cDNA using PCR. PCR conditions were optimized using Taq DNA polymerase in a 25µl reaction containing: 2.5µl of 10X PCR buffer, 25 mM MgCl2, 0.5 mM dNTPs, 2µl of each primer, 5 units of Taq DNA polymerase, 10 µl of template cDNA and 3 µl of nuclease free water. The reaction proceeded in a thermal cycler with the following conditions: 5 minutes at 94ºC, 40 cycles of 1 minute at 94ºC, 1 minute at 58ºC, 1 minute at 72ºC and final extension of 5 minutes at 72ºC. The PCR amplified product was sequenced at Macrogen Inc. (Seoul, Korea) after purification by JET quick PCR Product Purification Spin Kit (Genomed).

Phylogenetic analysis

The 18S rRNA gene sequence was aligned by using ClustalW while Nucleotide Blast (BlastN) was used to find out the similarity with already reported sequences present in databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Neighbor-joining (NJ) tree construction was done using MEGA 5 software to establish genetic lineage of studied sequence with reference one.

RESULTS AND DISCUSSION

Citrus leaves of healthy plants as control (Figure 1) and infected plants with black spots presenting the symptoms of fungal infection (Figure 2) were selected for the present study.



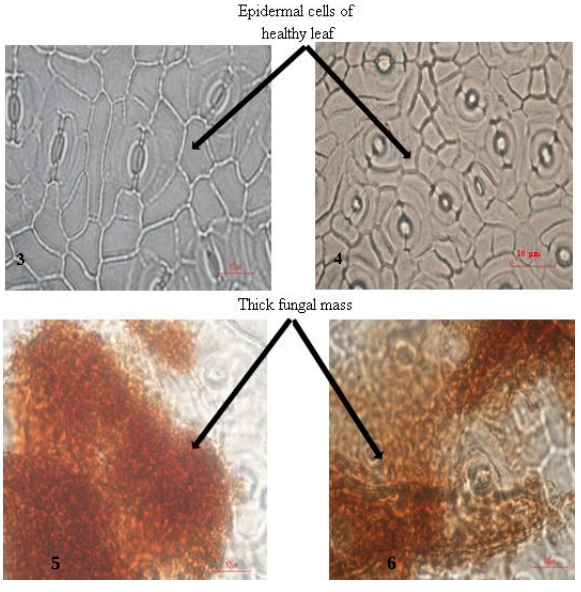
Figures 1-2: Leaf of citrus: **1.** Dry healthy leaf of citrus. **2.** Dry infected leaf of citrus with fungal mass on surface.

Microscopic analysis

Microscopic analysis of healthy leaves from adaxial and abaxial surfaces did not show any symptoms of fungal infection, as cells and stomata were very clear on epidermal layers (Figures 3 and 4). However, microscopic analysis of infected leaves of citrus in comparison with healthy leaves revealed that the epidermal surface of infected leaves was covered by a thick mass of fungus on both (adaxial and abaxial) surfaces (Figures 5 and 6). It was also revealed that fungus did not penetrate the cells rather it formed bulky structures on adaxial and abaxial epidermal layers of leaves.

This observation is in accordance with the findings of previous studies, as it was reported that the fungal species belonging to sooty molds group do not penetrate the cells and grow on the leaf surface, thus prevent the sunlight from

entering into leaf and result in reduced photosynthesis [16]. Our finding supports the hypothesis that an increase in the growth of this fungus results in reduction of photosynthetic efficiency and ultimately results in low yields of citrus production [3]. So, micro-morphological evidences supported the presence of *S. spongiosa* on citrus in Pakistan.



Figures 3-6: Microscopic epidermal view of healthy and infected citrus leaves at 100 X magnification power: **3.** Adaxial surface of healthy leaf. **4.** Abaxial surface of healthy leaf. **5.** Adaxial surface of infected leaf. **6.** Abaxial surface of infected leaf.

Molecular characterization

Total RNA was extracted from leaf tissues and amplified through RT-PCR using universal primers. Gel electrophoresis revealed specific amplification product of approximately 480 bp from infected leaf sample. However, healthy leaf sample did not show any amplification product. Blast search results showed that the studied sample is *S. spongiosa* which has 96 % homology with already reported 18S rRNA gene (GU214696.1) of that fungus. The 18S rRNA gene sequences obtained in this study has been deposited in GenBank with JX974604 accession number. This is the first report of the presence of *S. spongiosa* in Pakistan.

Phylogenetic analysis

Neighbor-joining (NJ) method was used to generate phylogenetic tree for representative aligned sequences by using MEGA5 [17]. Comparisons of plant species or gene sequences in a phylogenetic perspective can provide the most evocative understandings of biology [18]. Furthermore, phylogenetic analysis was carried out to find the evolutionary relationship of our studied sequence with available sequences of *Scorias spongiosa* in GenBank.

The phylogram (Figure 7) shows distinct dichotomy and can be divided into two major clades: clade I and clade II. Clade I included two isolates from Thailand and USA which form monophyletic group. These isolates showed close homology with each other. Early branching pattern in tree clearly indicated that the history of *S. spongiosa* is very ancient in USA [15]. Tree topology of the unrooted tree indicated the bifurcation of the internodes with symmetrical branching structure that demonstrated the even pattern of evolution throughout the tree topology. However, clade I acted as progenitor of clade II which can be further divided into two clusters (Cluster I and II) depending upon the sequence divergence. The present studied strain (JX974604) laid in clade II and showed close homology with strains from China (clade II, cluster I). Moreover, in clade II investigations revealed that strains from USA and Netherland showed close resemblances with each other by clustering together (Cluster II). Comparative analysis among reference sequences showed an equal mutation rate in all the strains from USA, Thailand, China and Netherland. Higher homology of our strain with Chinese HM480490 strain showed common ancestry.

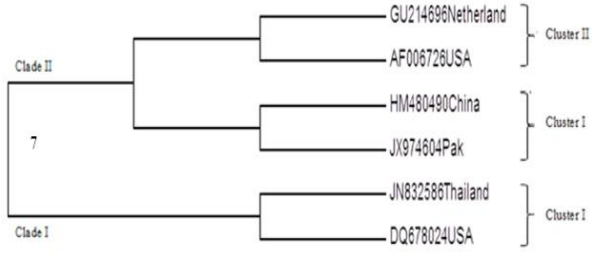


Figure 7: Phylogenetic tree by NJ method for available sequences 18S rRNA of *Scorias spongiosa.*

In this study due to higher homology of Pakistani strain with Chinese HM480490 strain it can be hypothesized that our strain might have been descendant from China due to migration event. The presence of *S. spongiosa* is well documented in China on bamboo plants [19]. However, this pathogenic fungus is originated from North America [15]. Moreover, it can also be hypothesized that strain of this fungus has been transported from America to China and then to Pakistan both accidentally and deliberately, through human activities [20]. The present study is an agreement with previous report which supports that travelling across the borders plays an important role in dissemination of diseases [21]. Majority of the pathogenic fungi are cosmopolitan in nature which migrate from one continent to other

through above mentioned routes. This fact is proved by the presence of black rot on citrus which was originated in eastern North America but now it occurs in Europe, South America and Asia [22]. It has been investigated previously that late blight of potato originated in Central America [23] and at present this disease is damaging potato crops throughout the world including Pakistan [24]. In this regard it can be concluded that *S. spongiosa* may have transported from China to Pakistan through one of such above mentioned patterns. Although, it seems to be host specific on an American beech tree when discovered, but later on it has been found in several plant species (including *Coffea arabica*, *Citrus sinensis*, *Eriobotrya japonica* and *Ehretia cyimosa*) in Kenya and on bamboo in China. However, in present study *S. spongiosa* is reported on citrus. So, the host range of this fungus demands more deep study. Our finding suggests that more intensive screening is necessary by using molecular techniques to provide further insight into mycological community in Pakistan.

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