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IDENTIFICATION OF THE CAUSAL AGENT OF SOOTY STEM AND BRANCHES WILT DISEASE IN SOME TREES IN IRAQ

Eman K. Abdul-Karim and Neran S. Aljarah
Plant Protection Department , Collage of Agricultural Engineering Sciences , University of Baghdad , Iraq .

ABSTRACT

The study was conducted at the College of Agricultural Engineering Sciences-Department of Plant Protection in 2018-2019 Baghdad/Iraq. The research aims to molecular identification of the fungal pathogen *Neoscytalidium sp.* the causal agent of the wilt branches disease on pomegranate, mulberry, apple, castor, and rubber by 18S ribosomal RNA gene and DNA sequencing, to demonstrate the pathogenicity, and host range of the fungal isolates. Five fungal isolates have been recoverd from the infected branches belonged to the five types of trees such as; apple (*Malus domestica*), pomegranate (*Punica granatum*), mulberry (*Morus alba*), india rubber (*Ficus elastica*), castor (*Ricinus communis*). The results showed compatibility of 100% with a standard in Gene Bank from *Neoscytalidium novaehollandiae* in pomegranate, mulberry, apple, and rubber, while in Castor having nine Trinsvertion C>G, A>T, T>G, T>G, G>C, T>G, A>C, A>T, and T>A, and five Transition G>A, G>A, A>G, A>G, and G>A) having 97% compatibility with standard in Gene Bank .This is the first record of the pathogen , *N. novaehollandiae* on these host trees in Iraq. The pathogenicity test confirmed their ability to infected all types of trees with different symptoms were observed, the results also, showed difference in infection severity which was found to be higher on the apple trees(88.09%).This result indicates the more susceptibility of apple trees to fangal sooty stem disease compared to other trees.

***Keywords*:** *Neoscytalidium novaehollandiae,* 18S ribosomal RNA gene, PCR Sequencing.

Introduction

The black rot or sooty stem disease was first reported globally in the peach and apricot trees in Egypt (Nattrass, 1933). The pathogen fungi has several names; *Hendersonula toruloidea*, *Scytalidium hyalinum*, *Nattrassia mangiferae* or *Neoscytiladium* sp. The main symptoms include wilted branches of the walnut trees (Wilson, 1947) and apricots (Calavan and Wallace, 1955). Recently, the fungus was classified belong to Botryosphaeriaceae family, which includes several plant pathogens considers as saprophytic and parasitic species that distributed globally with the genetic variations (Slippers and Wingfield, 2007; Paim *et al*., 2012). As a result of the technology development, we recommend using PCR technique, an accurate and rapid tool, for identifying several plant pathogens, including fungi (Fang and Ramsamy, 2015). PCR technique depends on the ITS1 and ITS2 or ITS1, ITS4, which helps to understand the evolution of fungi and the emergence of new species or strains (Iwata *et al*., 2006). Several regions of ITS have been identified in DNA, which is considered as a universal code for distinguishing fungi strains (Conrad *et al*., 2012). To classify the genera of Botryosphaeriaceae family, ITS cloning region are currently considered (Schoch *et al*., 2012). Pavlic *et al*. (2008) identified seven genera, including *N. noaehollandiae* from the *Adansonia gibbosa* in Western Australia using ITS and ∞-EFI. Furthermore, Mayorquin *et al*. (2016) used ITS in addition to B-tubulin and ∞TEF-1 to diagnose the species *N. hyalinum*, *N. noaehollandiae* and other fungal species that cause canker on the citrus trees in California.

Materials and Methods

Isolation and morphological identification of the pathogen

The pathogenic fungi were isolated from the following-infected trees; apple, mulberry, castor, rubber and pomegranate, located in Baghdad. The infected branches (samples) of each tree type were placed in bags with the date, time of collection and the type of trees. The samples brought to the plant disease labrotary. The infected branches were cut off ,from the healthy parts close to the affected area , into smaller parts (0.5 cm), sterilized, by immersing them in 20% of sodium hypochlorite solution for 3 minutes. The small pieces of branches were washed with sterile distilled water 3 times and dried with sterile filter paper. After that, they were transferred and planted onto petri dishes with Potato Dextrose Agar (PDA) supplemented with 50 mg/l of streptomycin sulfate to inhibition the bacteria growth.

All agar plates were incubated for 4 days at 25 ± 2 ºC. The pure colonies of fungi were isolated and transferred into a new plate and their phenotypic properties were reported. The identification of the causal agents of sooty or black rot fungi was based on the classification keys of the filamentous fungi (Seifert *et al*., 2011). The pure five isolates were identified according to the properties of the colony morphology that observe by the naked eyes, such as the colony color and the nature of fungal hypha (mycelium) growth. The spores were harvested, after inoculation into PDA and incubated at 25 ± 2 °C for 1 week. Ten milliliters of distilled water was added to each colony in petri plates and

collected in a sterile tube. The two diameters of 50 spores of different types were measured separately using the compound microscope (CX21FS1, Philippines).

The Molecular Characterization:

Total DNA of Fungi isolates was extracted by using ZR Fungal/Yeast/Bacterial DNA Mini Prep Kit (D6005/ZYMO/ USA). The 18S ribosomal RNA (ITS) gene was amplified using the primer F (5'-TCCGTAGGTGAACCTGCGG-3') and R (5'-CTCCTCCGCTTATTGATATGC-3') (Xie *et al*., 2008). The PCR amplification is performed in a total volume of 25µl containing 1.5µl DNA, 5µl Taq PCR PreMix (Intron, Korea), 1µl of each primer 16.5 pmol then nuclease-free water is added into a tube to a total volume of 25µl. Thermo cycling conditions were as follows: initial denaturation at 3 min at 95°C, followed by 35 cycles of denaturation 95 °C for 45 sec, annealing at 55°C for 1 min, extension at 72 °C for 1 min and a final extension of 72°C for 7min. The PCR products were separated on 1% agarose gel (onda/ USA). The gel is left to run for 60min with a 70volt/65Amp current. Following electrophoresis, visualization was conducted with a UV transilluminator after red-safe staining.

Pathogenicity test :

In This experiment, a small trees of two-year nursery growth were purchased from the nursery in Baghdad and planted at the College of Agricultural Engineering Sciences/ University of Baghdad. The distance between every two trees was 30 cm. The pure culture of the pathogenic fungi, which was isolated from on PDA, was inoculated into the healthy tree of 2 years old, from the same type of the tree previously had sooty rot and dieback symptoms. The inoculation sits was done 30 cm above the soil level to each stem, sterilized externally by wiping it with 10% commercial Clorox solution, cutting was made into the cortex tissues 10*2 mm (length * width) by sterilized razor. A disk (taken from the edge of the fungal growth for 4 days) was placed into the wounded sites covered with a small piece of wet cotton wrapped with aluminum foil to avoid the dryness of fungi growth (Lewis and Vanarsdel, 1978). The control group in this experiment was inoculated with a disk of PDA-free of fungal growth. Three wounds were made in each stem 20-22 cm distance between each two, three trees (replicates) for each trees type. The treated and control groups of trees were left for 8 months after inoculation. Due to the variation in the symptoms that observed in the treated trees, the following measurements were established to suit the symptoms noticed as following:

1-Symptoms of the wilting and sooty stem with the same diameter (figure 1) as in the control group (no pathogen). These were observed on apple, mulberry, rubber, and castor trees. The measurement of the canker area based on the length * width. The longest and the widest length were measured. The infection severity % was calculated as the lesion area according to the scale:

0- A healthy seedlings (no infection).

1- The affected area does not exceed 100 mm².

2- The affected area does not exceed 200 mm².

3- The affected area does not exceed 400 mm².

4- The affected area does not exceed 800 mm².

5- The affected area does not exceed 1600 mm²

6- The affected area is more than 1600 mm²

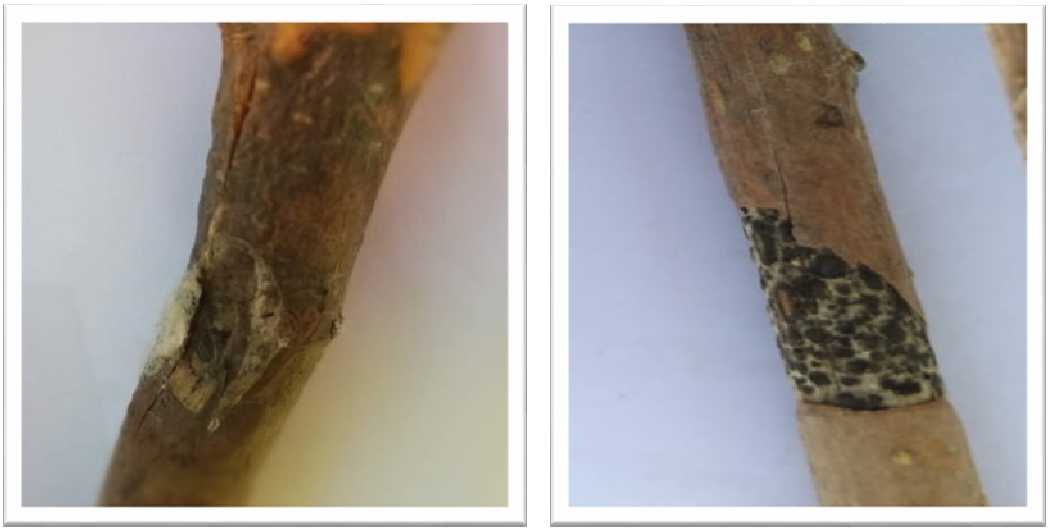

Fig. 1 : Symptoms of the wilting and sooty

2. Symptoms of semi-oval thickening galls in the stems of pomegranate trees (figure 2). These thickening galls extended along with the inoculation area and they are wider than the original of the trees' stems. So, the size of the thickening galls (size of the oval region) was calculated according to the following equation:

$$\text{The size of thickening galls} = \frac{4}{3}\pi abc$$

When (a) represents the large diameter, (b) the small diameter, and (c) the height of galls.

Fig. 2 : Symptoms of semi-oval thickening galls in the stems

The infection severity was assessed according to the following scale:

0- No infection.

1- The size of the thickening does not exceed 1 cm³.

2- The size of the thickening does not exceed 2 cm³.

3- The size of the thickening does not exceed 4 cm³.

4- The size of the thickening does not exceed 6 cm³.

5- The size of the thickening does not exceed 8 cm³.

6- The size of the thickening is greater than 8 cm³.

The percentage of infection severity was calculated according to Mckinney (1923) using the following equation:

Percentage of infection severity =

$$\frac{\text{The total No. of infected branches of each grade} * \text{its degree}}{\text{No. of testing branches} * \text{highest degree of infection}} \times 100$$

4. The host range of fungal pathogen isolates

A cross-inoculation was made among the five fungal isolates from five types of trees. The modified inoculated method that was described by Lewis and Vanarsdel (1978) used. The inoculated trees were left for 90 days (Sharma *et al*., 1984). Each experiment was conducted in triplicates. The severity of the infection was calculated based on the scales, as explained in the above.

Results and Discussion

1. Isolation and morphological identification of the pathogen

The isolates were characterized by their white colonies, at the beginning of their growth, and by time they converted to olive color, then to black. The changing of colors is due to the aggregation of fungal arthrospores in branched chains, also a visible density in the fungal hype was observed, these specifications conformed to many studies (Sutton and Dyko, 1989; Crous *et al.*, 2006). The microscopic test showed three types of spores; (i) the spherical with a diameters ranged between 3.3 and 11 microns, (ii) the unicellular elongated spore which was 5.5 - 11 microns in length and 3.3 - 11 microns in width and (iii) the bi-cellular which its length ranged between 6.6 - 12.1 microns and with width 2.2 - 6.6 microns. According to the previously listed characteristics, the fungus identified as *Neoscytalidium novaehollandiae*. A previous study was performed by Pavlic *et al*. (2008) showed that the isolated spores were 5.5 - 7.5 microns in length and 3.5-4.5 microns in width. *N. novaehollandiae* was first isolated by Pavlic *et al* (2008) in Australia from the baobab trees as well acacia, oak, manga, and ficus trees (Pavlic *et al*., 2008; Ray *et al*., 2010). Symptoms of the disease appear, represented by wilting of the branch, die back, canker spots on the leaves, gumination and death of trees (Nurul Nadiah, 2017). Based on the phenotypic characteristics, several fungi species could be identified and classified belong to Botryosphaeriaceae family (Taylor *et al*., 2000), as all fungi that are classified with morphological features (Leslie *et al*., 2006). These fungi species showed a significant genetic variation (Paim *et al*., 2012).

2. Molecular identification of pathogen

Compatibility of 100% in Gene Bank of 18S ribosomal RNA gene under sequence ID: MK064153.1, and have number score (933, 930, 928, and 791) bits, and expect (0.0),so no recorded change noticed from the Gene Bank in *Neoscytalidium novaehollandiae* in pomegranate, mulberry, apple, and india rubber.

The amplification of 18S ribosomal RNA gene for *Neoscytalidium novaehollandiae in* castor having nine Trinsvertion C>G, A>T,T>G, T>G,G>C,T>G,A>C , A>T, and T>A in locations (97, 132, 133, 139, 147, 148, 170, 175, and 249 nucleotide) respectively ,and five Transition G>A,G>A, A>G,A>G, and G>A in locations (125, 159, 184,194, and 267 nucleotide) having 97% compatibility with standard in Gene Bank as shown in (figure 3). under sequence ID: MK064153.1.

Score	Expect	Identities	Gaps	Strand
746 bits(827)	0.0	436/451(97%)	0/451(0%)	Plus/Plus

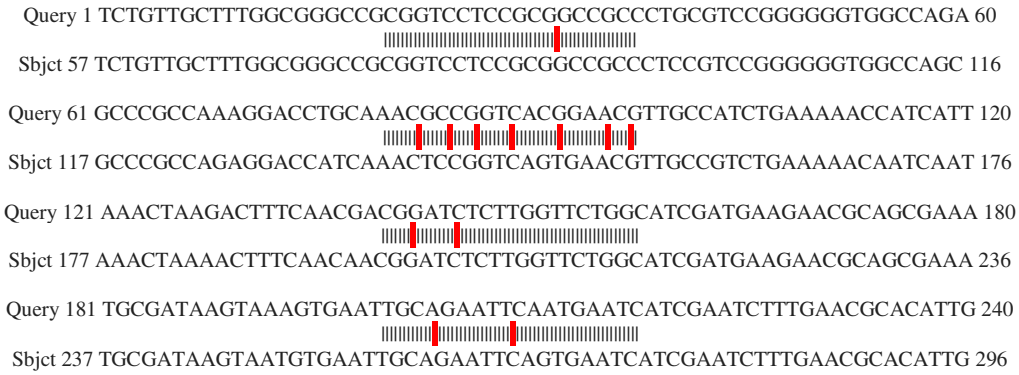

Fig. 3 : Alignment analysis of 18S ribosomal RNA gene. Query represents from the sample; Subject represents a database of National Center Biotechnology Information (NCBI).

The phylogenetic tree diagrammatic by Molecular Evolutionary Genetics Analysis (MEGA) software version 6.0 the phylogenetic trees of these species are shown in (figure 4) Neighbor-joining tree was constructed for phylogenetic analysis. These alignments appeared the *Neoscytalidium novaehollandiae* between Iraq and other global strains by partial sequence similarity in 18S ribosomal RNA gene for translating specific region. Hierarchical cluster analysis determine the following clusters including *Neoscytalidium novaehollandiae* Iraq isolate(1) the identical 100 % it is close to Turkey (ID: MK056265.1) the identical 100 %, while the *Neoscytalidium novaellandiae* Iraq isolate(2,3)Iraqi isolate the identical 98, and 97% respectively it is close to India (ID: MH428006.1), and China: Fukang(ID: HQ845383.1) the identical 98, and 97 %., also the *Neoscytalidium novaehollandiae* Iraq isolate(4,5)Iraqi isolate the identical 99 and 97 %respectively it is close to Iran: Izeh (ID: ID: KY449011.1),and Iraq: Kerbala (ID: MF511047.1) the identical 99,and 97 %.

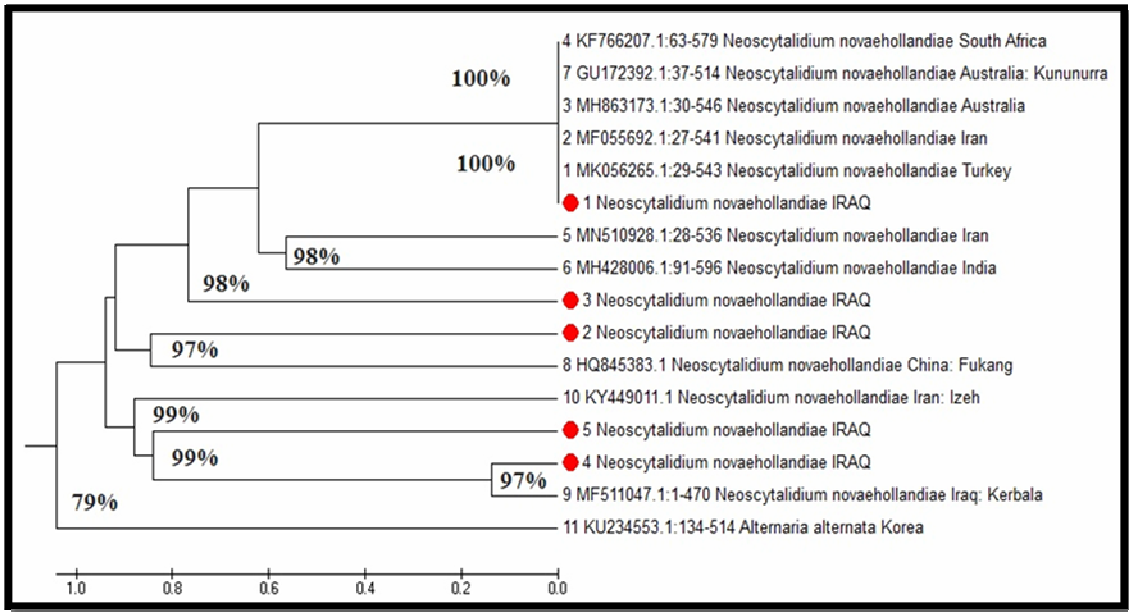

Fig. 4 : Neighbor-joining tree *Neoscytalidium novaehollandiae* of 18S rRNA gene. our isolates marked with red points

In the present study diagnose *Neoscytalidium novaehollandiae* by 18S ribosomal RNA gene for the wilting disease branching using PCR sequencing the samples were sent to Macrogen company and analysis by ABI 3730xl genetic analyzer (Applied Biosystems, US). Homology search was conducted using Basic Local Alignment Search Tool(BLAST) program which is available at the National Center Biotechnology Information (NCBI)online at (http://www.ncbi.nlm.nih.gov) and BioEdit program. The constant change in the taxonomy of fungi that belong to the genus *Neoscytalidium* has generated confusion; they have been named *Dothiorella mangiferae, Exosporina fawcettii, Fusicoccum arbuti, F. eucalypti, F. dimidiatum, Hendersonula agathidia, H. cypria, H. toruloidea, Scytalidium dimidiatum, S. lignicola or Torula dimidiata* (Farr *et al*., 2005; Schoch *et al*., 2014) *N. novaehollandiae* was isolated by Al-Kaabi (2019) from the fig trees and has been Molecular identification Recently, the studies used sequencing of the 18S rRNA region, it has been possible to recognize the species *N. novaehollandiae, N. orchidaceous, N. dimidiatum* and *N. oculus* (Phillips *et al*., 2013; Calvillo *et al*., 2019).

3. Pathogenicity test:

The table 1 data Indicate that all the five isolated fungi have the ability to infect various hosts such as; apple (*Malus domestica*), pomegranate (*Punica granatum*), mulberry (*Morus alba*), india rubber (*Ficus elastica*) and castor (*Ricinus communis*). However, the level of the symptoms and infection severity were vary.

The symptoms (cankers, sooty stem, wilting, and death) were observed at the inoculated branches in the apple, mulberry, castor and India rubber trees 3 months after inoculation (figure 1). In the infection area, a semi-oval thickening galls and enlargement was observed and increased in the diameter of pomegranate stem trees. The different symptoms that observed after fungal infection with different species of plants may be due to the pathogen-plant interactions or may be to the different species of the original trees which the pathogen were isolated, as well as the different health status of the trees. The symptoms of fungal infection of pomegranate could be related to the high quantities of secondary compounds, such as phenols and anthocyanin which may be plays an important role in increasing the plant's resistance to pathogens (Opara *et al*., 2009).

Our data showed a significant difference in the infection severity between apple trees (98.13%) and pomegranate trees (59.20%) when inoculated with their own fungal isolates. The difference in pathogenic effect of the fungal isolates on different species of trees could be related to variation in production of fungal toxins or enzymes with different quantities. Odibo *et al*. (1992) in their study referred to the capability of pathogenic fungi to produce enzymes, such as isoamylase which hydrolyzing starch and amylopectin, used as nutrients and a source for energy. Enzyme production, cellulase and lactase, by fungi isolates play a key role in cell components (Saleem *et al*., 2012 b; Devi *et al*., 2012). Al-Kaabi (2019) mentioned that the severity of the infection is related to the enzymatic production efficacy of fungal isolates in addition to the suspcetible of the trees themselves, or to the Interaction between pathogenic fungi and the trees.

Table 1 : The infection severity of *Neoscytalidium novaehollandiae* infection causing wilt branches and the sooty stem of different typs of trees after 3 and 8 months of inoculation in field condition.

Trees type	Infection Severity after 3 months of inoculation	Infection Severity after 8 months of inoculation
Pomegranate	59.2	66.6
mulberry	70.33	100
Apple	98.13	100
India Rubber	74	100
Castor	70.3	100
Control	0	0
LSD 5%	9.59*	0.043*

- Three branches from Each tree (host) was inoculated with fungi spp. (isolated previously from same infected trees). A 5 mm disk of pathogen pure culture of isolate was grown for 96 h on PDA.
- The branched of trees at 2-year old were inoculated into a certain wound in cortex tissue (length of 1 cm and depth of 1-2 mm) with fungi. Results were reported after 3 and 8 months of inoculation.
- The average of 3 readings per each trees and 3 trees per genus were calculated.

The results table 1 explained that the infection severity of the inoculated trees ,with the same isolates that previously isolated from them, increased significantly after 3 and 8 months of inoculation into apple, mulberry, castor and India rubber trees. The pathogenic symptoms include occurrence of cracks in the bark of mulberry trees and emergence of black spores beneath them, in addition to increase in the lesion size of the seedlings' branches and death of a large number of apple, mulberry, castor and India rubber trees. The severity of the infection reached 100% of above mentioned seedlings spp. while no significant change was observed after infection of pomegranate trees, in which infection severity were 59.2 and 66.6% after 3 and 8 months of inoculation in field condition. in he pomegranate trees, callus formed at the inoculation site (figure 2), which reduced the infection severity. This my be due to produce a secondary metabolic compounds work on reduction of infection severity and prevention of spreading infection in the plant.

4. The host range of fungal pathogen isolates:

The results in table 2. shows the infection severity of the five types of trees used in this study, confirm the wide host range of the five *N. neovaehollandia* isolates and no specific host was observed. The highest average of infection severity was reported in the Apple isolation (75.14%) compared to the lowest in the castor isolation (67.4%) after three months of inoculation with five fungal isolates in field conditions. The difference in the infection severity into various genus of trees could be due to the defensive responses of each tupe of trees toward the fungal isolates, as well as the quantitative and qualitative of enzymes/ toxins produced by the fungal isolates. The mean of infection severity rate was 88.09% in apple, with high significant differences with other trees. The lowest infection severity was noticed in the pomegranate trees with a significant differences compared to other host trees. This result shows the susceptibility of apple trees to infection with all *Neoscytalidium novaehollandia* isolates, which reflects the compatibility between the apple trees and the pathogen isolates. The pathogen isolates were recovered from the inoculated sites, while no pathogen was isolated in control treatment.

Table 2 : Infection severity of different tree typs to infection with *Neoscytalidium neovaehollandia* isolates after 3 months of inoculation in field condition.

Host tree	% of infection severity of pathgen isolated from fungal isolates					Average of infection severity
	Pomegranate	mulberry	Apple	Rubber	Castor	
Apple	85.130	85.130	98.130	86.970	85.100	88.092
mulberry	70.330	70.330	70.330	68.470	61.070	68.106
Pomegranate	66.600	51.830	61.070	61.230	51.830	58.512
Castor	79.570	74.030	81.430	75.870	81.430	78.466
Rubber	62.930	62.930	64.770	74.000	57.370	64.400
LSD 5%	2.607					1.166
Average	72.912	68.850	75.146	73.308	67.360	
LSD 5%	1.166					

- Each plant (host) was inoculated with fungi spp. (isolated previously from same and/or different infected plant spp. of plant). A 5 mm disk of pure fungal isolate was grown for 96 h of inoculation on PDA.
- The branched seedlings at 2-year old were inoculated into a certain incision (length of 1 cm and depth of 1-2 mm) with fungi. Results were reported after 3 months of inoculation.
- The average of 3 readings per each tree and 3 trees per genus were calculated.

The possession of fungi virulence genes, which are corresponding to the resistance and sensitivity genes of the host plant. Also, presence of suitable conditions for fungus-plant interactions could lead to the absence of fungal infection specialization toward a certain plants (Agrios, 1997). Providing a suitable condition for fungal growth could reduce and weaken the plant resistance to the pathogens and their enzymes/toxins which work non-specifically leading to wilting of tree branches which causes plant death. These observations were confirmed by several studies Karim (1987) and Al-Qassab (1999).

The difference in the fungal infection severity among the host plants could be related to plants' defense mechanisms and their production of secondary metabolites that play essential role in plant's protection against pathogens. On the other hand it could be the host vigor at the infection time appears to be a determined factor to induce canker and sooty stem in trees. Our data were in agreement with several studies that confirmed the broad host range of the pathogen to cause infection in different trees including; peaches, grapes, cassava, citrus, ficus, palm and melon (Namsi, 2010; Al-Saadoon, 2012; Machado *et al*., 2014; Mayorquin *et al*. 2016; Rahiminiya *et al*., 2018; Mirtalebi *et al*., 2019).

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