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FIRST RECORD OF LEAF BLIGHT AND TWIG DIEBACK CAUSED BY *STEMPHYLIUM SARCINIFORME* ON *CONOCARPUS ERECTUS* AND EDUCED A NOVEL DISEASE SEVERITY SCALE

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Abstract

Conocarpus erectus (Combretaceae). Introduced cultivar in Iraq and widely cultivated due their uses as folk remedy in anemia, catarrh, conjunctivitis, gonorrhea, diabetes, diarrhea, fever, headache, bleeding, tumors, orchitis, pricklyheat, swellings, and syphilis and for other purposes like their wood is used for fenceposts, crossties, turnery, boat building, firewood and landscaping purposes. because it can thrive, high tolerant temperatures .In 2016-2017, a severe foliar blight and twig dieback disease was observed in Babylon province ,Iraq. Disease incidence was up to 100%. The aim of this study detected the pathogen responsible of leaf blight and twigs dieback disease. The frequency of fungi isolated from necrotic lesions were: *Stemphylium* sp.(87%) and *Alternaria alternata*.(10%), *Aspergillus niger* and *Penicillium* sp.(3%). The pathogenicity test was checked based on application Koch's postulates, shown that *Stemphylium* was the primary pathogen caused the leaf blight and twig dieback disease of *C. erectus*. This study educed a novel disease severity scale for leaf blight and twigs of *C. erectus* caused by *Stemphylium sarciniforme* for the first time.

Key words: *Conocarpus erectus*, leaf blight, *Stemphylium*, disease severity scale.

Introduction

Conocarpus erectus (Combretaceae) commonly called buttonwood or button mangrove (Bashir *et al.*, 2015). This species grows on shorelines in tropical and subtropical regions around the world. Locations it is known from include USA, western Africa and Asia. It was introduced in the Gulf region and Iraq because it can thrive, tolerant of drought and high temperatures and absorbs brackish water.

The plant is used as folk remedy in anemia, catarrh, conjunctivitis, gonorrhea, diabetes, diarrhea, fever, headache, bleeding, tumors, orchitis, pricklyheat, swellings, and syphilis (Duke and Wain, 1981; Irvine, 1961; Morton, 1981). The leaves are eaten, or their decoction drunk, for fever (Irvine, 1961). Wood is used for fencepost, crossties, turnery, boat building, firewood and landscaping purposes. *C. erectus* is usually a dense multiple-trunked shrub, 1–10 m tall. They are dark green and shiny on top, and paler with fine silky hairs underneath, and have two salt glands at the base of each leaf. Stevens *et al.* 2001).

Unfortunately, no previous data available on this disease in Iraq, Gulf region and through the world, this study aim to explore the pathogen(s) causes leaf blight and twig dieback symptoms on *C. erectus*, as preliminary study based on traditional and molecular diagnosis.

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Materials and methods

Sample collection

This study was conducted between 2016-2017. A total of 200 samples of leaves and stems of *C. erectus* were collected randomly from different roadside trees and house gardens planted with *C. erectus* in Babylon province, located around geographical coordinates 33° 20' N 44° 24' E in the middle of Iraq, soil pH ranges from 6.6 to 8.8 (Guest , Al-Rawi ,1966). Plant parts were collected in polyethylene bags labeled with leaflets included date, collection site and other essential information.

Isolation and Identification of pathogens:

Isolation and purification of dubitable Bacterial and

Fungal isolates attacked *C. erectus* shoot: Adoptable infected leaves and twigs were sterilized using sodium bicarbonate at a concentration of 3.2% for two minutes and then washed triple times with sterile distilled water twice drying on filter paper, then about 1-1.5cm of 4 sterile leaf segments cut from lesion sites for each sample was transferred to Petri of Potato dextrose Agar (PDA) and nutrient agar for each by sterile forceps, then incubated the plates at 28 ± 2ºC for one - five days, followed detected the bacterial and fungal growths for each .any microbial growth were picked up by sterile loop into the new culture medium. Identified fungal isolates were incubated on PDA slant cultures were prepared from pure cultures dubitable as fungal pathogens and preserved at 4ºC for following purpose (Imran, 2011).

Identification of the fungal isolates

The pure culture of fungal isolates was subjected for colony characters identification and microscopic examination for conidial and conidiophore characters following Simmons (1990;1992).

Proven the potential causal agents for the disease in *C. erectus*.

In order to identify and characterize potential causal agents responsible for the disease outbreak in *C. erectus* populations, The identified fungal isolates were tested for their capacity to cause disease symptoms using the following inoculation procedure: the mid leaves of *C. erectus* plants were washed with sterile water triple times before the application disc inoculums of actively growing fungus cultures to the a biaxial surface (Schuck *et al.*, 2014). Disease symptoms caused by *Stemphylium sarciniforme* detached leaves. Agar plugs containing *S. sarciniforme* and *Alternaria alternata* culture were placed on detached leaves of *C. erectus* and incubated at high humidity. Detached leaves incubated with pure PDA plugs were used as control. Three replicates were used for each fungal isolate. Photos were taken after 3 and 5 days from inoculation period of *S. sarciniforme* and *A. alternata* isolates, respectively.

Genomic DNA extraction

The genomic DNA was extracted by using Phenol: Chloroform. The pure cultures of *Stemphylum* were frozen for 1 h. Tiny portions of the mycelia mat were harvested from frost colony and immerged into 1.5 ml tube, Based on Imran and Ali, (2012).

PCR assay

The following pairs of primer targeting ITS1-5.8S-ITS2 was used for PCR amplification (ITS5/ITS4). and amplified using thermal cycler PCR System (Labnet, USA) according of Imran and Al-Rubaey (2014) and Imran *et al.* (2016).

Construction of Disease severity scale

Many leaves and twigs were collected randomly from infected trees of *C. erectus* by *S. sarciniforme* .the leave were reclassified based on number of lesions and death degree in to seven categories (scales) from healthy one to completely dead leaves and twigs to educed disease severity scale.

Results and Discussion:

Preliminary pathogen screen test

In the first and crucial phase of the study, procurement and identification of the fungal and bacterial isolate from the leaves of *C. erectus*. The results shown no bacterial species was grown on infected leave segments on nutrient agar, only fungal growth was observed with no bacterial growth, the metabolites of *C. erectus* caused inhibition of bacterial species (Bashir *et al.*, 2015). The results revealed that fungal isolates associated with leave and twigs of *C. erectus* grown directly from lesions and from the mid vein of the leaves (Fig. 1). The results shown that pathogen used the vein as pathway for growth and production it toxic secondary metabolites the causes tissue necrotic (Imran and Al-Jopory, 2009; Schuck *et al.*, 2014).

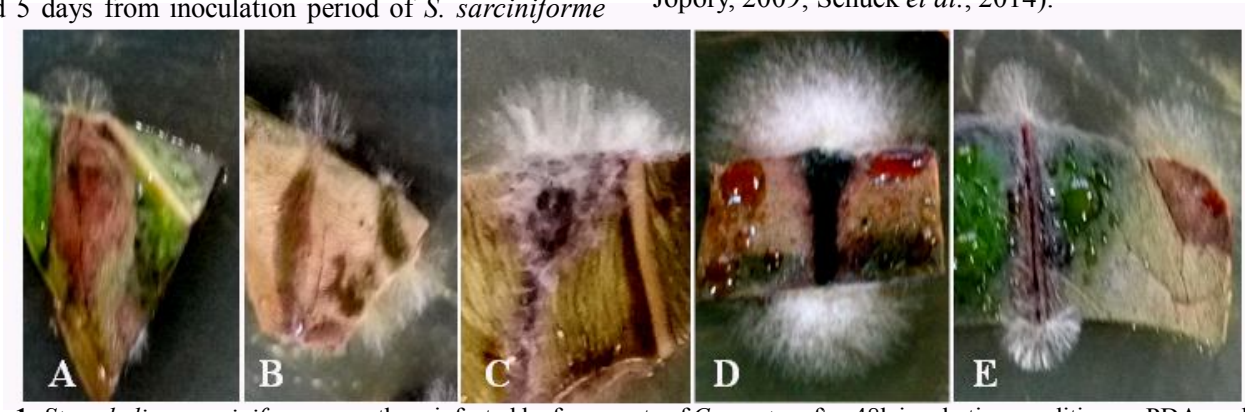

Fig. 1: *Stemphylium sarciniforme* growth on infected leaf segments of *C. erectus* after 48h incubation condition on PDA medium. Fungal growth from mid vein and necrotic lesion (A-E).

Isolation and identification of fungal pathogens

Two highly frequent fungi were grown on leaf lesions of *C. erectus*, isolated and diagnosed, these fungi are: *S. sarciniforme*.(87% frequency) and *Alt.alternate* (10% frequency), other fungi *Aspergillus niger* and *Penicillium* sp. (3% frequently). To identify the isolates under concern, microscopically characterization was used as has been previously utilized in various other studies (Schuck *et al.*, 2014). Unfortunately ,the results obtained in our study also support the facts non enlightened by previous studies that *S. sarciniforme* is an influential fungus, associated enormously with *C. erectus* plants in Iraq neither through the world.

The *S. sarciniforme* attacked leaves and whole twigs and causes dieback symptoms, resulted in over 90% mortality in some of plants roadside tree of *C. erectus*. The disease development in the roadside trees was illustrated in fig. 2.

Despite a population-wide slight increase in disease incidence and symptom severity (Fig. 3), at binging of March of 2016 and 2017, all the attacked plants were observed to shown death symptoms. All apical and terminal buds, whole leaves fail down due to severity of disease, but a new lateral buds were grown at together with the astonishing high capacity to recover from some symptoms at middle of April.

Fig. 2: View the severity of Leaves Blight and Twig Dieback disease on *C. erectus* plants(A-D), Caused by *S. sarciniforme* on the roadside trees.

Unfortunately, no previous reports about the fungal diseases attacked *C. erectus* through the world. As well as, this disease is uncommon in the *C. erectus* for landscape use in Iraq. Such disease-promoting conditions could have been created by the weather situation a few weeks prior the first survey of diseased plants: for several days in a row, lowering temperature were occur and sporadic rainfalls created an unusually cool and moist environment in the native habitat in winter of 2016 and 2017. Such environmental conditions participate be conducive for infection for a variety of fungal pathogens (Weber 2003; Pulimood *et al.*, 2007; Kriss *et al.*, 2010; Fourie *et al.*, 2013).

Development of disease in *C. erectus* trees:

This study was projecting the light on how the *S. sarciniforme* pathogen development on leaves and twigs of *C. erectus* through the Healthy plant fig. 4(A); the lesions caused leaves curling as in fig. 4(B): the disease rapidly development causing damaged more than half of plant as in fig. 4(C), at the final stage of disease development whole plant body (leave and twigs) became brown and dry and ˜100% plant leaves and twigs undergo death as in Fig. 4(D).

The Disease severity scale on *C. erectus*:

This study educed a novel disease scale of disease severity on leaves blight and twigs of *C. erectus* caused by *S. sarciniforme* for the first time (Fig. 5).

Fig. 3: Leaf blight and twigs dieback symptoms of *C. erectus* caused by *Stemphylium sarciniforme*.

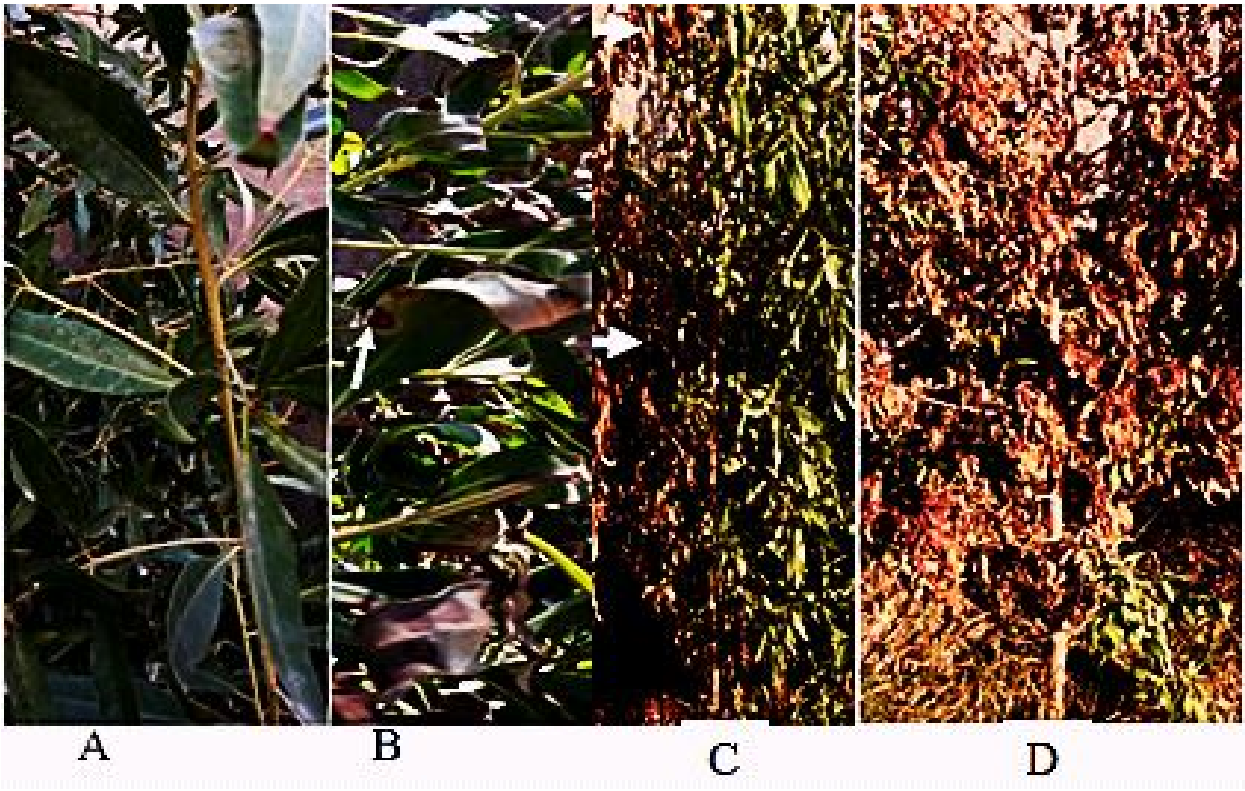

Fig. 4: Development of leaf blight and twigs dieback disease on *Conocarpus erectus*: A: Healthy plant, B: the lesions caused leaves curling, C: damaged more than half of plant, D: whole plant body (leave and twigs) became brown and dry.

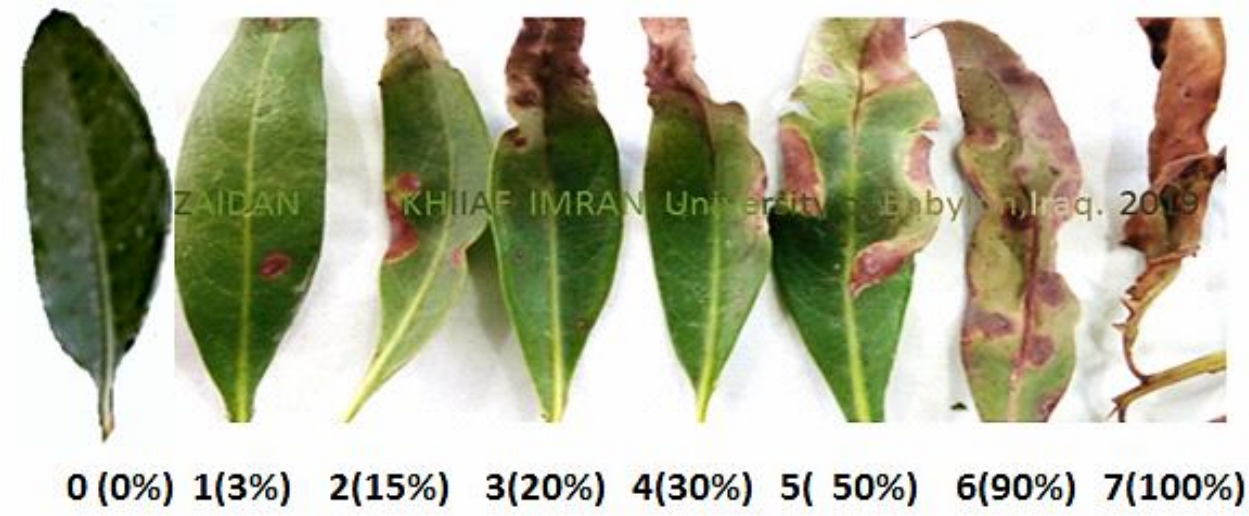

Fig. 5: Scale of Disease severity of Leaves blight and twigs dieback on *C. erectus*.

0 - No lesions per leaves (healthy leaves)

1 - one small leaf lesions (3% infection)

2 - Few lesions on few leaves with no stem lesions (15% infection).

3 - Few lesions on few leaves or with apical buds of stem lesions (20% infection).

4 - Many large leaf lesions and twigs dieback (30% infection).

5 - Most plants leaves death (50% infection)

6 - Most of plant leaves and twigs death (90% infection)

7 - Whole plant leaves and twigs death (100% infection).

Calculated virtual example for severity based on our scale:

$$Disease severity(\%) = \frac{Sum\ of\ Disease rating}{Total\ no.of\ rating \times Maximum scale} \times 100$$

Microscopic characterization of the native fungal isolates

Disease scale	No. of Leaves (rating)	Disease ratin
0	8	0
1	20	3
2	12	15
3	10	20
4	9	30
5	7	50
6	5	90
Max. Scale = 7	4	100
Total	**75**	**308**

$$\text{Disease severity (\%)} = \frac{308}{75\times7}\times100 = 0.58.7\times100 = 58.7\%$$

In addition, the native fungal isolates were also characterized microscopically by analyzing the sporulation and conidial shapes from 5-7 day-old fungal cultures under the light microscope. Typical conidia shape, color, length and septa numbers were compared with original description of *S. sarciniforme*, *Conidia phragmospore*, they were multicellular with several vertical and transverse septa (Fig. 6). Conidia color was pale brown. This species is not reported on *C. erectus*, but not found on any trees and shrubs worldwide including.

Fig. 6: MICROSCOPIC FEATURE OF *Stemphylium sarciniforme* observed by light microscopy conidiophores, Conidiogenous cell and conidia. Scale bar 25µm.

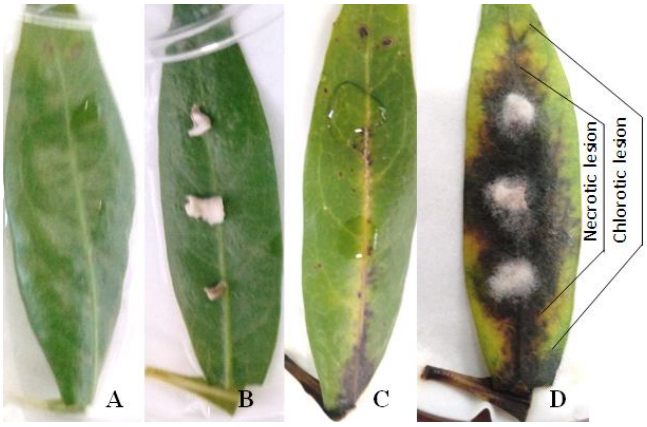

Fig. 7: Evaluation of *Stemphylium sarciniforme* pathogenicity using detached leaf assay.

Development of bioassays using *C. erectus* and its fungal pathogens

To assess the virulence of the individual fungal isolates on *C. erectus* plants, a detached leaf assay was performed by placing agar plugs with fungal cultures on excised *C. erectus* leaves (Fig. 7). The differences in virulence of the native fungal isolates were evaluated based on average diameters of colour change from green to dark-brown, necrotic and hypersensitive-like lesions. *S. sarciniforme* isolates were the first ones to cause visible symptoms in form of hypersensitive-like lesions after three days of incubation (Fig. 7). The lesions expanded rapidly from the inoculation points and started to overlap each other already two days later. Chlorotic lesions were not detected for *S. sarciniforme* isolates.

Therefore *A. alternata* strains were defined as being less aggressive isolates. This experiment helped to assess the virulence of each native fungal isolate necessary for choosing the appropriate candidates for further bioassays (Schuck *et al.*, 2014).

The Agar plugs containing *S. sarciniforme* culture caused the largest necrotic/hypersensitive-like lesion on detached *C. erectus* leaves while the moderate isolates caused visible necrotic/hypersensitive-like lesions only slightly larger than the diameter of pure agar plugs (control) (Schuck *et al.,* 2014).

PCR assay results

Both the internal transcribed spacer region (ITS1, 5.8S, and ITS2) and flanking regions of primers binding sites of rDNA gene of two isolates from the original lesion isolation. The length of the amplified rDNA region shown 685 bp shown good quality PCR amplification for achieve identification of *S. sarciniforme*. The PCR result indicating that these two fungal isolates belong to the genus *S. sarciniforme* (Fig. 8) (Imran *et al.* 2016).

Disease Impact

Brown leaf spot disease caused by *S. sarciniforme* can have a substantial impact on plant growth under favorable conditions. The relationship between the amount of leaf tissue affected by brown leaf spot and the amount of green area is unclear. However, the lesions reduce the amount of photosynthetic areas on leaves available to contribute carbohydrates to the developing shoot.

Obviously plant disease cannot continue to increase forever, and as the level of disease approaches 100%, the disease progress curve gradually flattens out. disease progress starts out looking linear but slows down as it approaches a maximum (Fig. 3, 4). The pathogens may also enter the host plant by penetration through a natural

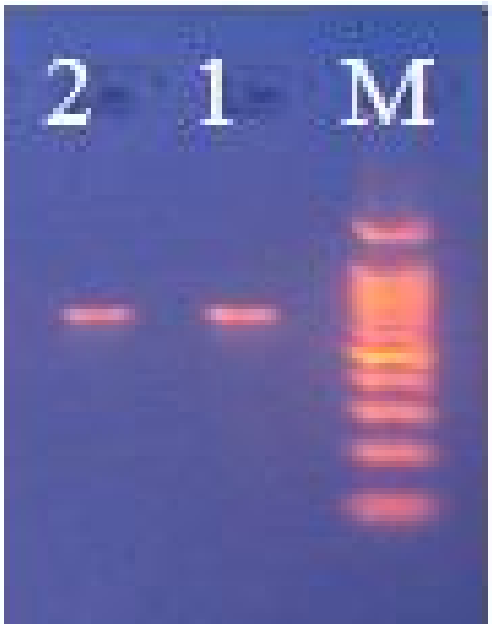

Fig. 8: Gel electrophoresis of ITS PCR products, amplified for two isolates of *Stemphylium sarciniforme* with (ITS5 / ITS4) primers. Lanes 1-2, DNA amplified M, molecular-weight markers (100 bp DNA ladder, Promega).

opening (like a stomata pore) or via a wound, the successful of pathogen correlated with the host and the external environment, or use direct penetration of the plant surface to enter the host via enzymatic degradation in order to overcome the physical barriers presented by the plant's surface. (Hamid and Strange 2000). Our results not enlighten on all disease properties due to the absence previous studies through the world, so this disease required for further studies.

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