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Molecular identification and artificial pathogenicity of *Fusarium solani*; the causal pathogen of wilt on *Centaurea ragusina* in Egypt

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Abstract: **In this study, the causal agent of *Centuarea ragusina* wilt disease was recorded for the first time in Egypt. Morphological characterization and DNA sequencing showed that the pathogen was *Fusarium solani*. A pathogenicity test conducted gave 60 % infection. A pure culture of *F. solani* was isolated on potato dextrose agar (PDA) at 25 °C from diseased plants and deposited in the culture collection of the Assiut University Mycological Centre as AUMC 9299 and the ITS sequence was uploaded to GenBank as accession number MG734215.**

***Keywords: Fusarium solani, Centuarea ragusina*, Asteraceae, wilt, AUMC.**

1. INTRODUCTION

Centaurea species are important medicinal herbs that had been extensively used in traditional medicine for many decades (Kaij-a-Kamb et al. 1992). It is the fourth biggest genus in the family Asteraceae, consists of 600 species around the world, especially in Mediterranean regions and as well as Western Asia (Garcia-Jacas et al. 2000). In Egypt, thirteen species of *Centaurea* are growing and distributed (Bakr et al. 2016). Keen interest in phytochemical and biological research had been directed to *Centaurea* species due to their excellent medicinal properties and the presence of diversity of bioactive phytochemicals and prevalence of flavonoids and bitter crystalline unsaturated lactones (Amigo et al. 1984; Öksüz and Ayyildiz 1986; Koukoulitsa et al. 2002; Flamini et al. 2004; Shoeb et al. 2007; Seghiri et al. 2009). *Centaurea* species also contain some other toxic compounds such as repin, subluteolide, janerin, cynaropicrin, acroptilin, and solstitialin which are involved in Parkinson's disease (Burrows and Tyrl 2001; Sanders et al. 2001; Gupta 2007) and nigrostriatal degeneration (Van den Munckhof et al. 2006). Investigations of *C. aegyptiaca* revealed the presence of guaianolide which showed potential cytotoxic activities against liver and larynx carcinoma cell lines (Sarg et al. 1987; Orabi et al. 2013).

Up to now, no records about *C. ragusina* wilt caused by *F. solani* in Egypt were found. So, the aim of this work was to identify the causal agent of *C. ragusina* wilt in Assiut governorate, Egypt, including morphological, sequencing and proof of its pathogenicity.

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2. MATERIALS AND METHODS

Isolation and morphological identification of the pathogen

In September 2013, wilt disease of *Centuarea ragusina* was noticed and recorded for the first time in Egypt near the plantation of Assiut University Mycological Centre in Assiut University campus, Assiut governorate, Egypt. Samples of the diseased plants were collected separately in sterile polyethylene bags and transferred promptly to the laboratory for examination and mycological analysis. The diseased stem parts were cut off into segments (3 cm in length), washed with tap water and then with 0.5 % sodium hypochlorite for 3 min, rinsed with sterile distilled water and placed on Petri dishes (9 cm) containing PDA and incubated for 7 days at 25 °C. The fungal colony appeared around the stem segment was isolated on PDA at 25 °C and preserved as pure culture at 4 °C for further investigation. The morphological identification was carried out at the AUMC according to Booth (1971) and Leslie and Summerell (2006).

Molecular identification of the pathogen

DNA extraction

DNA extraction was done according to Mohamed (2011). About 0.2 g of 4-day-old fungal mycelia of *F. solani* AUMC 9299, grown on PDA, were harvested and grounded in a sterilized, precooled mortar using liquid nitrogen and transferred to 1.5-ml microfuge tubes. Afterwards, a preheated 800 µl CTAB buffer (3 % CTAB, 1.4 M NaCl, 0.2 % ß-Mercaptoethanol, 20 mM EDTA, 100 mM TRIS-HCl pH 8.0, 1 % PVP-40) were added to each tube, mixed and incubated for 30 min at 65° C. 800 µl of CI Mix (24 ml chloroform, 1 ml isoamyl alcohol) were gently added and mixed to avoid genomic DNA sharing, followed by centrifugation for 10 min at 10000 xg. Afterwards, the aqueous phase was transferred into a new tube; this step was repeated several times to obtain a clear sample. For DNA precipitation 2/3 volume of a precooled at -20 °C isopropanol was added and gently mixed. The samples were incubated overnight at 4 °C, thereafter centrifugation for 10 min at 13000 xg. The supernatant was removed and the pellet was washed with 200 µl washing buffer (76 % ethanol and 10 mM ammonium acetate). The washing buffer was carefully decanted and the pellet was suspended in 200 µl TE buffer supplemented with RNase (10 mg/ml). After incubation for 30 min at 37 °C, 100 µl of 7.5 M ammonium acetate and 750 µl of ethanol were added and gently mixed. Samples were centrifuged for 10 min at 13000 xg at room temperature. Subsequently, the supernatant was completely removed and the pellet was suspended in a suitable volume of sterile distilled water.

PCR for rDNA and sequencing using ITS1 and ITS4 primers

PCR was carried out at the Molecular Biology Center, Assiut University, Assiut, Egypt. The universal primers ITS1 and ITS4 (White et al. 1990) were used. In the PCR tubes 1µl of DNA template, 1 µl 2.5 mM dNTP mix, 0.2 unit of Taq polymerase, 5 µl of 10x complete buffer and 40 µl of sterile ddH2O. 10 pmol of ITS1 (5´ TCC GTA GGT GAA CCT TGC GG 3´) and ITS4 (5´ TCC TCC GCT TAT TGA TAT GC 3´) were added. The whole reaction was denatured initially for 3 min at 94° C, thermocycled as following for 36 cycles: denaturation at 94 °C for 30 sec, followed by annealing for 30 sec, and extended for 1 min at 72 °C. Finally, the reaction extended 3 min more at 72 °C for proofreading and was holed at 4 °C. The PCR products were then purified prior to sequencing. Then the purified PCR products were reconfirmed (using size marker) by electrophoreses of the PCR products on 1% agarose gel. Then these bands were eluted and sequenced. Each sample was sequenced in the sense and antisense direction.

Contigs were created from the sequence data using DNA Star software version 5.05. The sequence obtained from each isolate was further analyzed using BLAST from the National Center for Biotechnology Information (NCBI) website. Sequences obtained with those retrieved from GenBank database were subjected to ClustalW analysis using MegAlign (DNA Star software version 5.05) for the phylogenetic analysis. Sequence data were uploaded to GenBank.

Pathogenicity test

The pathogenicity test was carried out in the greenhouse. The inoculum was prepared from a 7-day-old culture of *F. solani* AUMC 9299 grown in potato dextrose broth (PDB). The liquid PDB was filtrated and the density of *F. solani* spore suspension was adjusted to $1x10^6$ (spore/ml). 3-month-old seedlings of *C. ragusina* were uprooted from the soil, their roots washed with tap water, rinsed by sterile distilled water and dipped for 30 min in the spore suspension of *F. solani* (Bhat 2003). Seedlings were planted in plastic pots (20 cm in diameter) filled with sterilized sandy clay soil. Non-

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inoculated seedlings were dipped in sterile distilled water and used as a control. The development of symptoms of *Fusarium* wilt was monitored and the percentage of infected plants was calculated after 6 weeks of incubation compared with the control.

3. RESULTS

Wilt symptoms were observed in some individuals of *C. ragusina* near the Assiut University Mycological Centre in the Assiut University campus. The symptoms were monitored as yellow shoot, foliage faded to a whitish hue, and the plants dried up, stunted and died (Fig. 1). Fungal mycelia were grown out from affected tissues plated on PDA by day 4 after inoculation. Phenotypic characteristics revealed that the pathogen was *F. solani* (Fig. 2) and the molecular analysis based on sequencing of the ITS region confirmed this identification (Fig. 3). A pure culture of *F. solani* was deposited in the culture collection of the Assiut University Mycological Centre as AUMC 9299 and the ITS sequence was uploaded to GenBank as accession number MG734215

Pathogenicity test based on artificial inoculations led to the development of wilt symptoms on *C. ragusina*. In contrast, no signs or symptoms were noticed on the control plants. Symptoms developed on 60 % of *C. ragusina* plants from which *F. solani* was re-isolated (Fig. 4).

4. DISCUSSION

In this current investigation, wilt of *C. ragusina* is recorded in the Assiut University campus near the AUMC for the first time worldwide. The fungal pathogen was identified as *F. solani* based on both the morphological features observed in cultures and by sequencing the ITS region of the rDNA operon, followed by a phylogenetic analysis in which sequences obtained in this study were compared with those of known isolates. The same organism is known to infect a wide range of plants in varied climates worldwide. For example, *F. solani* was recorded as pathogenic to Trirave and Kaumera varieties of sugarbeet in Egypt (El-Abyad and Abu-Taleb 1991) as well as root rot disease in olive trees in Tunisia (Amira et al. 2017), bean root rot in Cameroon (Toghueo et al. 2016), chilli wilt disease of *Capsicum annum* in India (Sundaramoorthy et al. 2012), faba bean root rot in Ethiopia (Belete et al. 2013), dry rot on potato in Iran (Chehri et al. 2014) and foliar and root diseases on orchids (Benyon et al. 1996; Ichikawa and Heideki 1998; Latiffah et al. 2009; Chung et al. 2011; Srivastava 2014; Srivastava et al. 2018). The study of *F. solani* wilt of *C. ragusina* opens the door for scientists to pay more attention towards the control of this disease.

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Fig. 1: A&B, healthy *C. ragusina* plants; C&D, Symptoms of wilt disease caused by *F. solani* AUMC 9299

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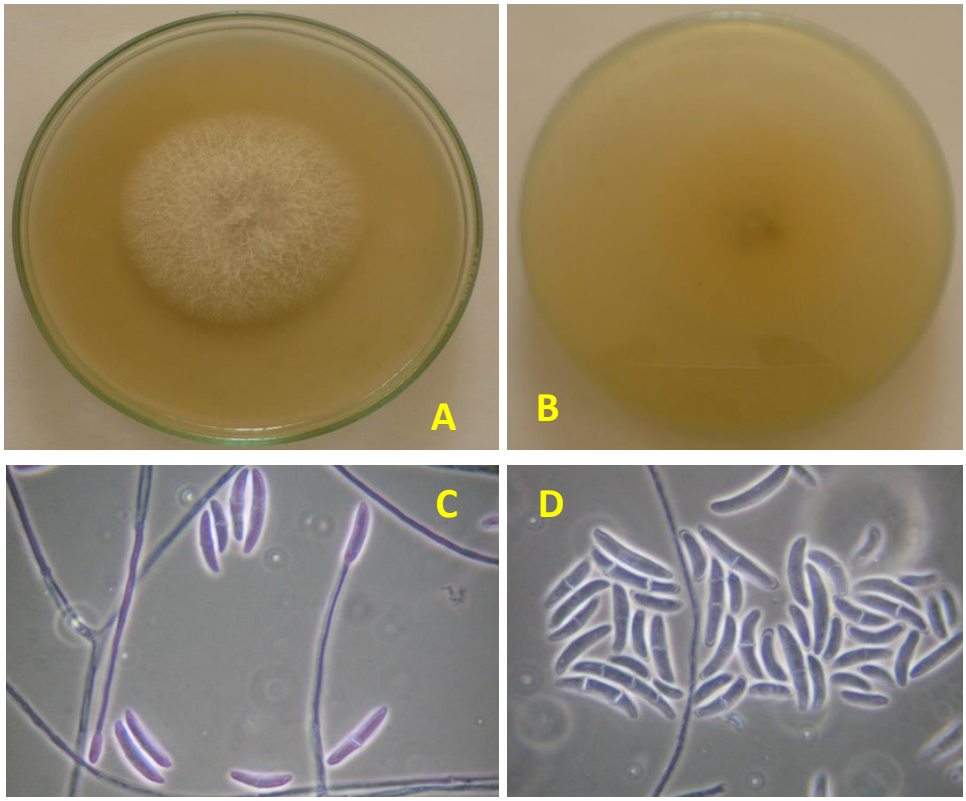

Fig. 2: A, 4-day-old colony of *F. solani* AUMC 9299 on potato dextrose agar (PDA) at 25 °C; B, reverse; C, characteristic long phialides bearing microconidia; D, macroconidia.

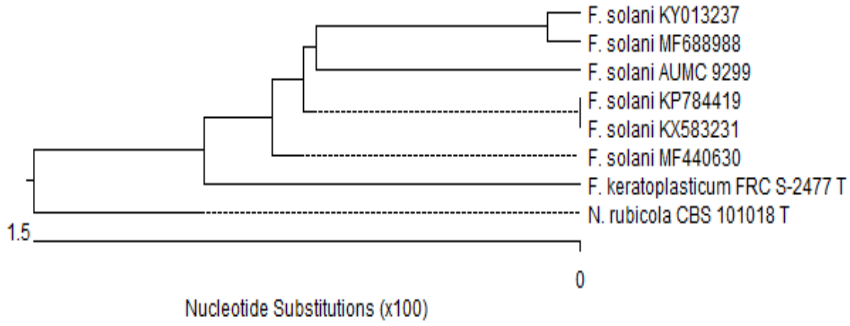

Fig. 3: Maximum likelihood tree based on sequencing of the ITS region of *F. solani* AUMC 9299 with the most similar sequences in GenBank.

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