



FIRST RECORD OF ROOT ROT OF ROSEMARY (*ROSMARINUS OFFICINALIS*) CAUSED BY *RHIZOCTONIA SOLANI* IN IRAQ

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Abstract

Rosmarinus officinalis, which commonly known as rosemary is one of the most economically important species of the family Lamiaceae. In October, 2017, root rot symptoms were observed on rosemary in gardens of Horticulture Department, Collage of Agriculture & Forestry, Mosul University, Ninevah governorate, North Iraq specifically, the leaf tissues were blighted and white mycelial growth was seen on the stems. The fungus was isolated from diseased tissue and cultured on potato dextrose agar for identification. The young hyphae had acute angular branching near the distal septum of the multinucleate cells and mature hyphal branches formed at an approximately 90° angle. This is morphologically identical to *Rhizotonia solani*. Pathogenicity of the fungus in rosemary plants was also confirmed by Koch's postulates. Molecular identification of *R.solani* isolate was done by amplifying the internal transcribed spacer (ITS) region of the conserved ribosomal DNA using primers ITS1 and ITS4. All the ITS sequences were compared for gaps and similarity sequences of the fungus were homologous to those of *R.solani* isolates in the GenBank database with a similarity percentage of 97%, thereby confirming the identity of the causative agent of the disease. The nucleotide sequence of ITS from the Iraqi isolate has been assigned GenBank Accession No MN 396663.1. Here, for the first time we report *R. solani* as the causal agent of root rot of rosemary in Iraq.

Key words : Root rot, *Rhizoctonia solani*, Rosemary, *Rosmarinus officinalis*.

Introduction

Rosmarinus officinalis, which commonly known as rosemary is one of the most economically important species of the family Lamiaceae. Rosemary, is a woody, perennial herb with fragrant, evergreen, needle-like leaves and white, pink, purple, or blue flowers, native to the Mediterranean region. The name "rosemary" derives from the Latin for "dew" (ros) and "sea" (marinus), or "dew of the sea". The plant is also sometimes called anthos, from the ancient Greek word ανθος, meaning "flower". The plant is now widely distributed all over the world mainly due to its culinary, medicinal and commercial uses including in the fragrance and food industries (Bozin *et al.*, 2007).

There are several diseases that may infect Rosemary plants, some which may be fatal for these plants and are often very difficult to get rid-off once they infect the shrub. One of the significant diseases is known as the root rot which can cause wilting in Rosemary plants and kill them in nearly all growing fields. This disease begins to decay and spread the rot to healthy roots, possibly killing the entire plant (Mirabolfathy and Ershad, 1993;

Garibaldi *et al.*, 2013). Fungal cases of root rot are caused by dormant fungus in the soil that takes hold when excess water is added to the plant, the common symptoms of disease caused by this fungus are sheath blight, leaf blight, root rot, damping-off, root rot, head rot and bottom rot (Duan *et al.*, 2008; Yang *et al.*, 2008). The fungus was reported to cause root rot of rosemary in Iran, USA, Spain, Italy, Korea and UK (Holcomb, 1992; Garibaldi, *et al.*, 3013), but has not been reported in Iraq so far. The aim of this research was to identify the causative agent of the root rot disease observed in rosemary in Ninaveh governorate, based on mycological characteristics and pathogenicity test and identify it using internal transcribed spacer (ITS) region of the conserved ribosomal DNA using primers ITS1 and ITS4.

Materials and Methods

• Fungal isolation: In October 2017, root rot symptoms were observed on rosemary in gardens of Horticulture Department, Collage of Agriculture & Forestry, Mosul University, Ninevah governorate, North Iraq specifically, the leaf tissues were blighted and white mycelial growth was seen on the stems. Diseased plants were collected

in sterilized plastic polythene bags and sent to the laboratory for pathogen isolation. Infected plant parts were cut into small pieces measuring about 1.0~1.5cm, surface sterilized with 0.1% sodium hypochlorite (NaOCl) for 1 min, washed thrice with distilled water and dried with a sterilized filter paper. The small pieces were then placed in petri dishes containing potato dextrose agar (PDA) medium and incubated at $25 \pm 2^\circ\text{C}$ for 5 days. To obtain a genetically pure culture, the hyphal tips from the mycelia grown on the PDA were cut and transferred to fresh PDA dishes

Nucleus stain method was also carried out for evident identification of *Rhizoctonia* species isolated from infected samples and soil around the roots (Sneh *et al.*, 1996). Two small wood pieces were placed on the filter paper on Petri dish and two lams were located on them, then a piece of mycelium was put on lams. The mycelium was grown and reached under the lams after 2 days, then lams were separated and their mycelium were pigmented with one drop of safranin 0.5% and one drop of fresh KOH 3% and visited by light microscope.

• Pathogenicity test: The pathogenicity of the isolate was confirmed by inoculating *R. officinalis* plants with *R. solani* fully colonised PDA plugs (5 mm). Plants were approximately 25 cm high and 5 months old at time of inoculation and grown in 3 Kg pots containing sterile soil. Five plants were inoculated with a colonised agar plug placed at the stem base just the soil line, another five plants were inoculated with agar plugs, as control. Plants were placed in a glasshouse and watered as required. After ten days, plants were removed from the soil and assessed for the presence of symptoms. The fungal pathogen was re-isolated from disease lesions of inoculated plants. The re-isolated pathogen met the criteria stipulated by Koch's postulates and exhibited the same morphological characteristics as those seen in the original isolates.

• Genomic DNA extraction and PCR amplification: Pure culture of *R. solani* was e grown in potato dextrose broth (PDB) for 10 days at 25-28°C in the dark. Mycelia were harvested by filtration through filter paper (Whatman No. 1). The harvested mycelia were used immediately for DNA extraction using Fungal/Bacterial/ Yeast DNA MiniPrep™, Catalog No. D6005 according to the manufacturer procedures.

• Agarose gel electrophoresis of DNA: Electrophoresis has been done to determine DNA pieces after the process of extraction or to detect the result of the interaction of PCR during the presence of the standard DNA to distinguish the bundle size of the outcome of the interaction of PCR on the Agarose gel.

• Prepare of the Agarose gel: According to Sambrook *et al.*, (1989), the agarose gel has been made in 1.5% condensation by melting 1.5g of agarose in 100 ml of previously made TBE buffer. Agarose has been heated to boil then left to cool down at (45-50°C). The gel has been poured in the pour plate in which the plate of agarose support has been prepared after fixing the comb to make holes that would hold the samples. The gel has been poured gently not to make air bubbles and left 30 minutes to cool down. The comb has been removed gently of the solid agarose. The plate has been fixed to its stand in the Electrophoresis horizontal unit represented by the tank used in the Electrophoresis. The tank has been filled with TBE buffer in which it covers the gel surface.

• Preparation of sample: 3 µl of the processor loading buffer (Intron / Korea) has been mixed with 5 µl of the supposed DNA to be electrophoresis (loading dye), after the mixing process, the process of loading is now to the holes of the gel. An Electric current of 7 v/c2 has been exposed for 1-2 h. till the tincture has reached to the other side of the gel. The gel has been tested by a source of the UV with 336 nm after put the gel in pool contain on 3µl Red safe Nucleic acid staining solution and 500 ml from distilled water.

• Detection of Gene *ITS* by Using PCR: Detection of *ITS* gene was conducted by using primers for amplification. A fragment of *ITS* was amplified using a forward primer (*ITS1* F: 52 - TCCGTAGGTGAACCTG CGG-32) and a reverse primer (*ITS4* R:52 TCCTCCGC TTATTGATATGC-32) (Primers set supplied by IDT (Integrated DNA Technologies company, Canada). The PCR amplification was performed in a total volume of 25µl containing 1.5µl DNA, 5 µl Taq PCR PreMix (Intron, Korea), 1µl of each primer (10 pmol) then distilled water was added into tube to a total volume of 25µl. The thermal cycling conditions were done as follows: Denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 45s, 52°C for 1 min and 72°C for 1 min with final incubation at 72°C for 7 min using a thermal Cycler (Gene Amp, PCR system 9700; Applied Biosystem). The PCR



Fig. 1: *R. solani* isolated from infected Rosemary showing multinuclear cell and right-angle branched hyphe.

Table 1: Represent Type of Polymorphism of 16srRNA Gene from *Rhizoctonia solani* Isolate.

Identities	Expect	Score	Sequence ID	Nucleotide	Location	Type of substitution
97%	1e-75	279	ID: FJ492168.2	C>T	247	Transition
				G>C	211	Transversion
				A>C	210	Transversion
				G>T	209	Transversion
				G>C	200	Transversion
				T>G	171	Transversion

products were separated by 1.5% agarose gel electrophoresis and visualized by exposure to ultraviolet light (302 nm) after red stain staining (Intron Korea).

- **Sequencing and Sequence Alignment:** The PCR products were separated on a 2% agarose gel electrophoresis and visualized by exposure to ultra violet light (302 nm) after Red Stain staining. Sequencing of gene was performed by national instrumentation center for environmental management (nicem) online at (http://nicem.snu.ac.kr/main/?en_skin=index.html), biotechnology lab, machine is DNA sequencer 3730XL, Applied Biosystem), 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

Results

- **Fungal isolation:** The fungus produced a white mycelium when young, but produced a brown mycelium with dark brown sclerotia after growing on PDA at $20 \pm 2^\circ\text{C}$ for 14 days. Each cell had more than two nuclei. The hyphae was 4-7 μm in diameter and mature hyphal branches formed at an approximately 90° angle (Fig. 1). This is morphologically identical to *Rhizoctonia solani* (Parmeter, 1970).

- **Disease symptoms:** Different soil-borne fungal species produced various wilting symptoms on the infected Rosemary plants. The main symptoms on

infected plants in the studied areas were yellowing, root rot, crown rot and stem canker on the health and wilted plant. Symptoms showed wilting on some parts of plants or death of whole infected plants (Fig. 2). Commonly, the disease caused remarkable yield reduction which resulted economical problem for producers.

- **Pathogenicity test:** Symptoms similar to the original symptoms were seen on inoculated plants leaves, whereas no symptoms were observed in control plants. The pathogenicity test was performed twice. The fungal pathogen was re-isolated from disease lesions of inoculated plants. The re-isolated pathogen from the inoculated and symptomatic plants was attempted. *Rhizoctonia solani* was isolated from the symptomatic plants thereby fulfilling Kochs postulates. No *Rhizoctonia* isolates were recovered from attempted isolations taken from pieces of the noninoculated plants. It was recognized as the causal pathogen of root rot of rosemary.

Root rot of rosemary caused by *R. solani* has been reported previously in the United States, India, Iran, Brazil, Italy, Korea and UK (Holcomb,1992; Garibaldi *et al.*, 2013; Aktaruzzaman *et al.*, 2015; Azcona *et al.*, 2017). Here, for the first time we report *R. solani* as the causal agent of root rot of rosemary in Iraq. To the best of our knowledge this is the first report of *R. solani* in Iraq.

- **Sequencing and Sequence Alignment:** To confirm the morphological identification, the internal transcribed spacer (ITS) region of the *Rhizoctonia* isolate was amplified with universal primers ITS1 and ITS4. The isolate was partially diagnosed after conformity with the copies at the gene bank at National Center Biotechnology Information (NCBI) genes gave 97% (diagnostic accuracy) match with isolation : [FJ492168.2](#) Query cover of the sequence was 97% Error Value 0.0. The results obtained showed that 6 variations: first C>T



Fig. 2: Symptoms showed wilting on some parts of plants or death of whole infected plants.

Transition and five Transversion variatins G>C, A>C, G>T, G>C and T>G have shown 97% compatibility as showed in table 1 and fig. 1.

Results showed the close genetic relationship among *R. solani* isolated in this study (indicated with black prism) and those worldwide deposited in genbank database. fig. 2,

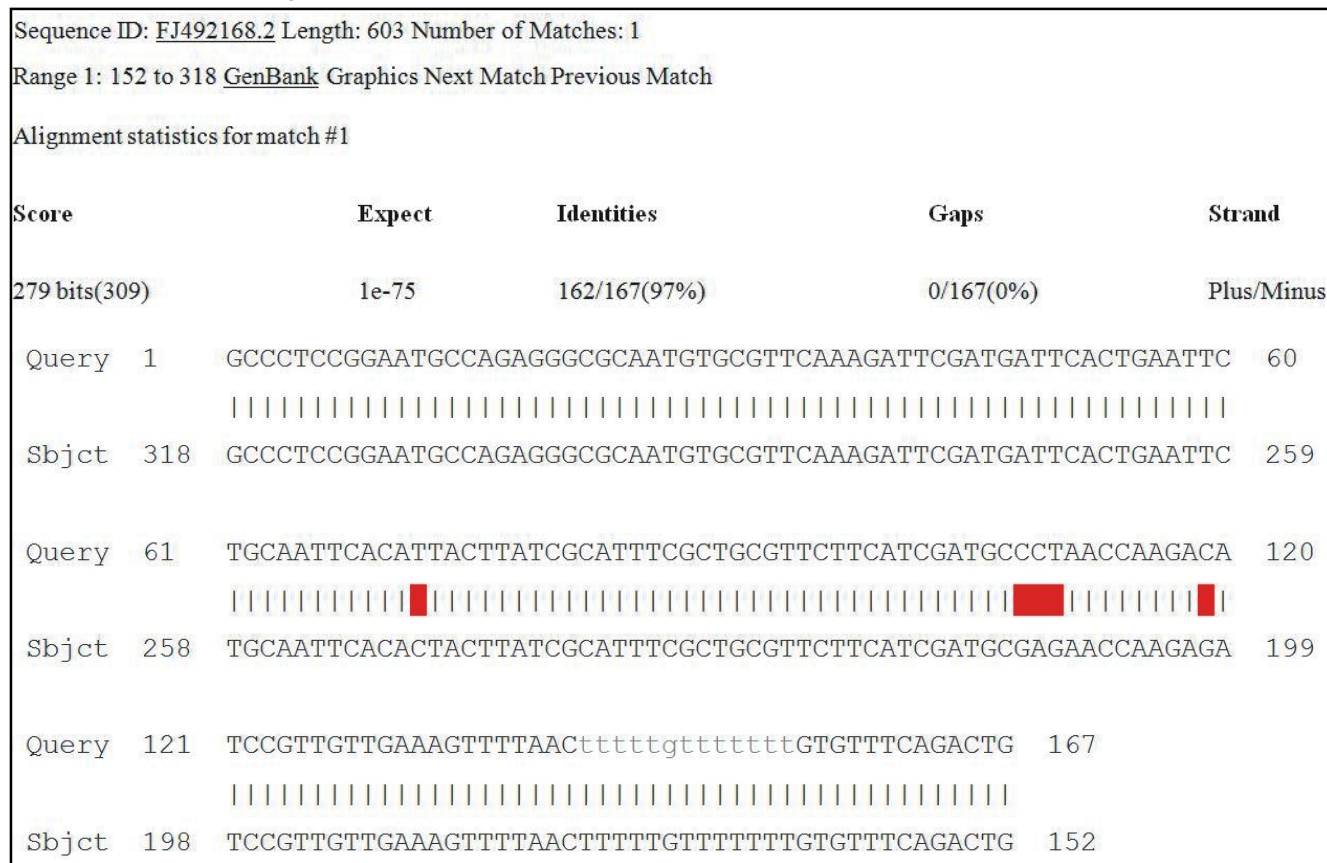


Fig. 1: Sequencing of sense flanking the partial *ITS* gene compared with standard : FJ492168.2 gene, obtained from Gene Bank. Query represents of sample; Subject represent of database of National Center Biotechnology Information (NCBI).

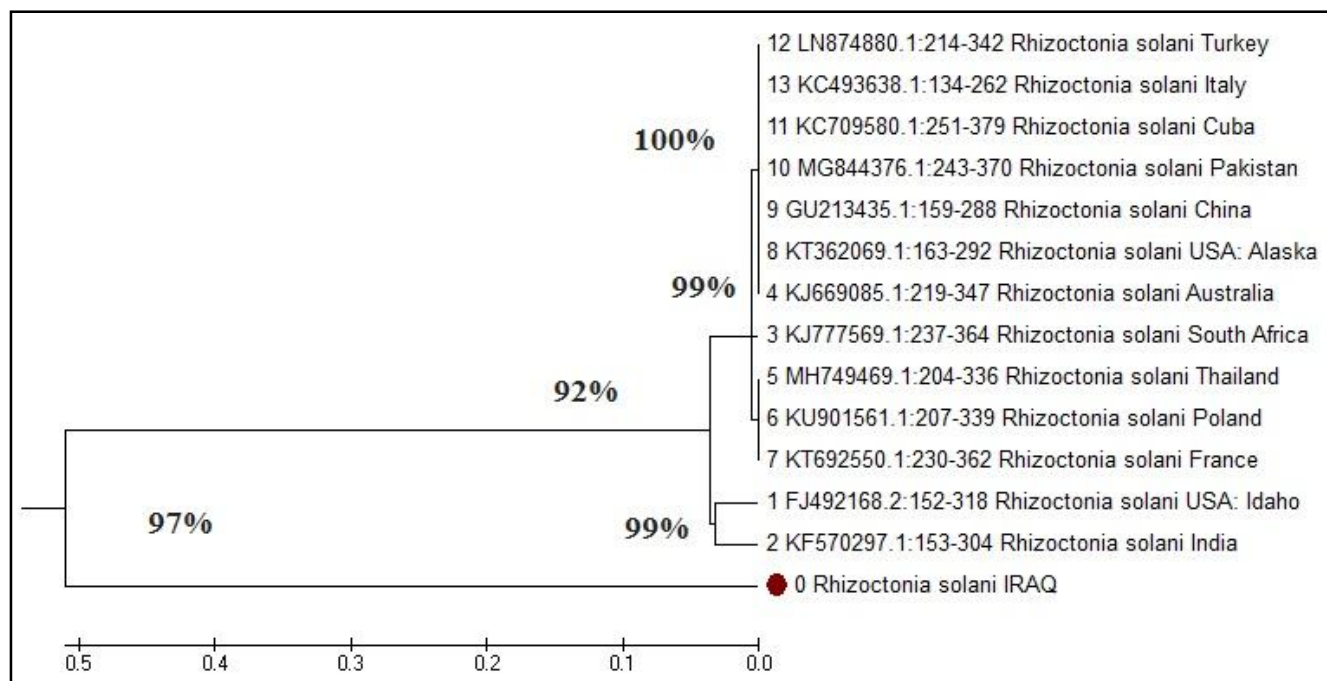


Fig. 2: Phylogenetic tree constructed by the neighbor-joining method showing the phylogenetic relationships of *R.solani* compared with the reference sequences from gene bank.

represents comparison between local Iraqi isolate strain of *R. solani* with the strain *R. solani* recorded in the National Center Biotechnology Information (NCBI) and isolated from different countries showed compatibility 97% with *R. solani* strains accession No. FJ492168.2 from USA: Idaho, 93% with accession No. KJ777569.1 from North Africa and 92% with accession Nos. KF570297.1, KJ669085.1 and GU213435.1 from India, Australia and China respectively and 91% with accession Nos. MH749469.1, KU901561.1 and KT692550.1 from Thailand, Poland and France respectively. The nucleotide sequence of ITS from the Iraqi isolate has been assigned GenBank Accession No MN396663.1. Here, for the first time we report *R. solani* as the causal agent of root rot of rosemary in Iraq.

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