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First record at molecular level for *Rhizoctonia solani* causing Rot Root on *Aleo vera* plants in Iraq

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ABSTRACT

Aloe vera is an important medicinal and cosmetic plant. This study aimed to isolate and diagnose the pathogenic fungi associated with infected Aloe vera in nurseries, Nineveh Province, Northern Iraq in 2020. The results showed the emergence of different pathogenic fungi isolated from the roots, leaves, and stem base (crown area) of Aloe vera. The primary diagnosis based on the morphological characteristics of the fungal colony grown on PDA showed the presence of *Rhizoctonia solani* with the highest frequency of 82.5%. *R. solani* showed different infection symptoms ranging from yellowing and leaf curling, roots in reddish brown colour, and the infection progresses resulted in appearance of reddish necrotic areas on the crown area. The fungal pathogenicity was confirmed using Koch's postulates. *Alternaria alternata* ranked second with a frequency rate of 55.45% followed by *Fusarium solani* with 43.75%. The root rot causing *R. solani* isolate was confirmed by molecular diagnosis using PCR technique. This is considered to be the first study in Iraq for diagnosing fungi on Aloe vera using PCR with pair of specialized primers Forward FITS 5′-TCCGTAGGTGAACCTGCGG-3′ and Reverse RITS 5′- TCCTCCGCTTATTGATATGC-3′. The results showed that the isolate under study belongs to *R. solani* with a band size of 550 pb, which is the expected size produced by these primers. The results of DNA sequencing of *R. solani* isolate under study was registered under accession number 1. MW737660. Based on the NCBI information, the isolate showed a similarity in the internal cloning region (ITS) with other isolates by 92-99%.

Keywords: *Rhizoctonia solani*, Fungi, Rot Root, Aloe vera.
Article type: Research Article.

INTRODUCTION

Medicinal plants have many healing and medicinal properties, beside their antioxidant activities (Rajeshwari & Andallu 2011) which made them to be a reliable source for manufacturing chemicals (Sheet & Ali 2011). Aloe vera is in the cactus family Asphodelaceae (Liliacea) and reproduces by cuttings and seeds. It is the oldest plant used for medicinal purposes. The historical records indicate that Aloe vera was used in the treatment of some diseases more than 6000 years ago, since the Roman era (Crosswhite & Crosswhite 1984). Aloe vera is characterized by containing antioxidant and antimicrobial compounds (Rajasekaran *et al.* 2006). The genus Aloe includes more than 300 species, the most important and most widely used medicinally (Nazar 2011). It is a succulent plant whose leaves are seated, broad, dense, green, fleshy, covered with a waxy skin. The height of the plant ranges between 60-100 cm (Panovska *et al.* 2005). Aloe vera leaves are characterized by its antioxidant and antimicrobial activities (Rajasekaran *et al.* 2006), containing a large number of mineral elements, especially potassium, calcium, sodium and magnesium, important in human health. In addition, it also contains twenty amino acids with relative amount of fatty acids, the most important of which are Olic, Linoleic, and Stearic (Tungala *et al.* 2011; Kumari *et al.* 2012). The plant is also a source antioxidation vitamins (A, B and C) especially vitamin A and C (Rajesh *et al.* 2012). Aloe vera is one of the few plants that contain vitamin B_{12} in addition to other active compounds such as minerals, lignin and saponins (Pankaj *et al.* 2013). The gel is one of the contents of Aloe vera leaves. It is a viscous, colourless liquid that contains several compounds, including anthraquinones, glycosides, glycoproteins, and prostaglandins. The latter is considered to be growth inhibitor of plant pathogenic fungi, such as *Aspergillus niger*, *Penicillum digitatum*, *Botrytis cinerea*, *Aspergillus flavus* and *Fusarium*

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solani, as well as viruses antagonistic (Uzma *et al.* 2011, Manvitha *et al.* 2014 & Georgakopouiou *et al.* 2020). On the other hand, Aloe vera was effective in stimulating growth of broilers and improving the productive capacity of treated birds (Singh *et al.* 2013). It was also found to have a positive effect on some physiological and biochemical blood characteristics of quail female (Raouf *et al*. 2016). Aloe vera is native to North Africa and Spain and is grown in hot and dry tropical regions of Asia, Europe and America (Manvitha & Bidya 2014). Commercially, China, United States of America, the southwest of the Arabian Peninsula and Saudi Arabia are the main producers of Aloe vera (Pankaj 2013). The increase in cultural and medical awareness and interest in the field of cosmetics in Iraq, the increasing opening of beauty centres in recent times, has led to the increase of nurseries for Aloe vera in specific areas, especially in the governorates of Anbar, Baghdad and Kirkuk. Aloe vera is exposed to many pathogens, including fungi (Shubhi Avasthi *et al.* 2011; Shaker 2016; Shubhi Avasthi *et al.* 2018; Muhammad *et al.* 2019) and bacterial (Goffin *et al.* 2013; Perves *et al.* 2016). Moisture levels also play an important role in increasing plant disease (Avasthi *et al.* 2018). There are also some reports about plant infection by fungi around the world (Karimi *et al.* 2017; Al- Abbasi *et al.* 2021; El-Sayed *et al.* 2022). The lack of studies on this medicinal plant in Iraq prompted this study, which aims to isolate and diagnose the fungi associated with Aloe vera, determining the most frequent and frequent pathogenic fungi on the plant, and focusing on the phenotypic and molecular diagnosis of the pathogenic fungus using Polymerase Chain Reaction (PCR). Thus, this is the first study at the molecular level of the Aloe vera in Iraq.

MATERIALS AND METHODS

Isolation and Diagnosis

Isolation of pathogens associated with aloe vera

Samples of Aloe vera (leaves, roots, and stem base), which showed different symptoms of infection, were collected from nurseries in Nineveh Province, Iraq. The samples were kept in clean plastic bags and transferred to the Plant Pathology Laboratory in the Plant Protection Department, College of Agriculture and Forestry, University of Mosul. The samples were washed with tap water for two hours, then plant parts (roots, leaves and the crown area) were taken, cut into small pieces approximately 4-5 mm. The pieces were superficially sterilized in 1% sodium hypochlorite (NaOCl) for 3 minutes, washed with distilled water for a minute and dried. The cuts were distributed on 9-cm Petri dishes containing sterile, chloramphenicol-treated (50 mg L^{-1}) PDA medium. The different plant parts were grown separately at a rate of 5 pieces per plate, and incubated at 25 ± 2 ºC for 5 days. Then, the isolates were purified and microscopic examination was conducted to investigate the associated fungi and calculate the percentage of recurrence of each fungus where:

$$\text{Fungal frequency (\%)} = \frac{\text{No. of pieces showed fungus}}{\text{Total No. of pieces in the sample}}$$

Phenotypic diagnosis

A portion from the edge of pure, newly-cultured fungal colony was taken, placed on a glass slide for microscopic examination. Distinguishing characteristics were identified in diagnosing the causative agent of root rot, stem base and leaf yellowing in Aloe vera. The taxonomic key of the fungus *Rhizoctonia solani* was used in determining phenotypic taxonomic characteristics on the basis of mushroom mycelium (septate or aseptate, branched or unbranched), spore composition, shape and texture of the fungal colony on the nutrient medium.

Molecular diagnosis of *Rhizoctonia solani* under study for Genomic DNA Extraction

Mycelium of *R. solani* isolated from Aloe vera was utilized instantaneously for the extraction of DNA while utilizing DNA MiniPrep™ Kit, Catalogue, D6005 (ZYMO, USA) according to the protocol of the manufacture company. Specialized primers for the ITS region (Internal Transcribed Spacer) were used according to the 18S ribosomal RNA gene for the control isolate ID: MW498394.1 identical at 99% (Table 1). For the PCR, genetic primers were prepared from Inter grated DNA Technologies Company, Canada for reaction according to manufacturer's instructions (Table 2). The reaction materials (Table 3) were prepared using the Maxime PCR PreMix Kit from the Korean company, Intron, which consisted of I-Taq DNA Polymerase, a mixture of dNTPs and a mixture of buffer solutions in one PCR tube. According to Sambrook *et al* (1989), the agarose gel was prepared. The sample was prepared for electrophoresis where

a 3-µL loading buffer (Intron, Korea) was mixed with 5 µL of the supposed DNA to be electrophoresis (loading dye). The PCR Product was subjected to electrophoresis, then the DNA concentration was determined (Table 5).

Table 1. RNA gene polymorphism of isolate from *Rhizoctonia solani.*

Gene: 18S ribosomal RNA gene						
No.	Type of substitution	Location	Nucleotide	Sequence ID with compare	Source	Identities
1	Transversion	477	C\A	ID: MW498394.1	*Rhizoctonia solani*	99%

Table 2. The specific primers of gene ITS used in the interaction to detect *Rhizoctonia solani.*

Forward	5′- TCCGTAGGTGAACCTGCGG -3′	60.3	50 %	550 base pair
Reverse	5′ TCCTCCGCTTATTGATATGC-3′	57.8	41 %	

Table 3. The Components of the Maxime PCR PreMix kit (i-Taq).

i-Taq DNA Polymerase	**5U µL^{-1}**
DNTPs	2.5 mM
Reaction buffer (10X)	1X
Gel loading buffer	1X

Table 4. The optimum condition of detection.

1	Initial Denaturation	95 °C	3 min	1 cycle
2	Denaturation -2	95 °C	45sec	
3	Annealing	52 °C	1 min	35 cycle
4	Extension-1	72 °C	1 min	
5	Extension -2	72 °C	7 min	1 cycle

Table 5. DNA sample concentration according to Nanodrop results.

sample ID	**Nucleic acid conc. (ng mL^{-1})**	**260/280 purity**
1	77	1.9

Sequencing analysis

The PCR products were separated on a 2% agarose gel electrophoresis and visualized using UVL (302 nm) after ethidium bromide staining. Sequencing of gene was performed by National Instrumentation Centre for Environmental Management (NICEM), online at (http://nicem.snu.ac.kr/main/?en_skin=index.html), Biotechnology Lab. Machine was DNA sequencer 3730XL, Applied Biosystem. Homology search was conducted using Basic Local Alignment Search Tool (BLAST) available at the National Centre Biotechnology Information (NCBI) online at (http:// www.ncbi.nlm.nih.gov) and BioEdit program.

RESULTS

Isolation and diagnosis

The results of laboratory isolation showed the occurrence of a number of pathogenic fungi isolated from different parts of infected Aloe vera plants. The isolated fungi were accompanied by various disease symptoms represented by yellowing and wilting, drooping leaves, rotting of roots and stem bases, and leaf spots. By repeating the laboratory isolation, it was noted that the most frequent fungus was *Rhizoctonia solani* associated with root and stem rot, which recorded a frequency of 82.2%, followed by *Alternaria alternata* with 55.55% and *Fusarium solani*. While the least fungi associated with infected Aloe vera was *Cliendero carbon* spp. with a frequency of 21% (Fig. 3).

Phenotypic diagnosis

The results of microscopic examination of the fungus isolated from Aloe vera infected with crown and root rot showed the appearance of a single pure brown fungal colony on the PDA food medium (Fig. 4). The diameter of the colony was 9 cm after 9 days of incubation at 25 ± 2 ºC. Microscopic examination of the developing colony at 40X magnification,

exhibited that the mycelium is divided with many branches at almost right angles with the main mycelium. The presence of transverse septa near the point of emergence of the branch with a clear narrowing in the areas of branching, and it is one of the taxonomic signs of the fungus *R. solani*. The septa between cells are perforated. These holes allow the movement of cytoplasm, mitochondria and nuclei from one cell to another, confirming the characteristics of *R. solani* (Simson *et al.* 2017).

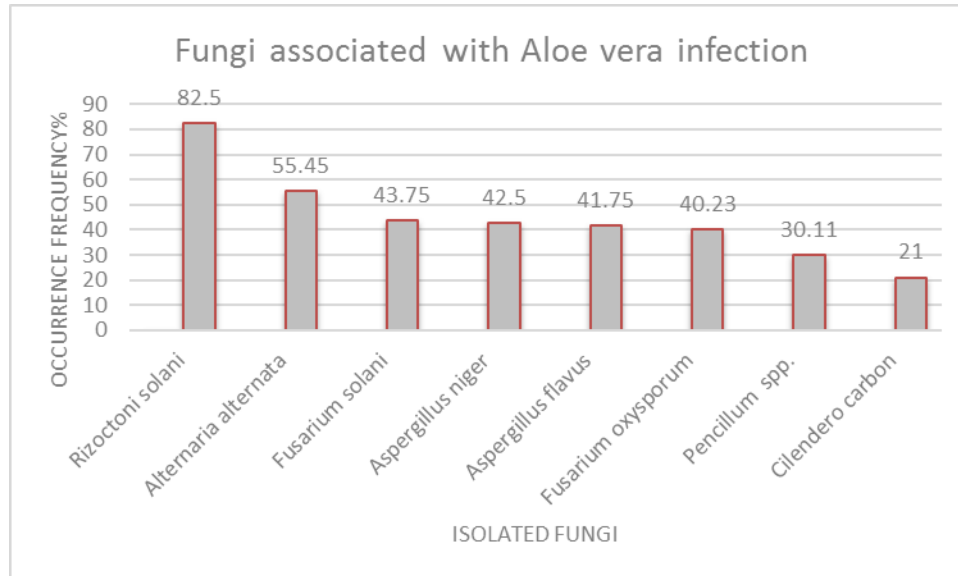

Fig. 1. Frequency percentage of fungi associated with infected Aloe vera plants.

Fig. 4. A. *R. solani* colony on PDA culture media, and B. fungal mycelium at 40X magnification**.**

Extraction of genomic DNA

Spectrophotometer results showed that the DNA density of *R. solani* isolated from infected aloe vera, produced DNA at a concentration of 77 ng mL^{-1} with a purity of 1.9 which was used for subsequent PCR reactions (Fig. 5).

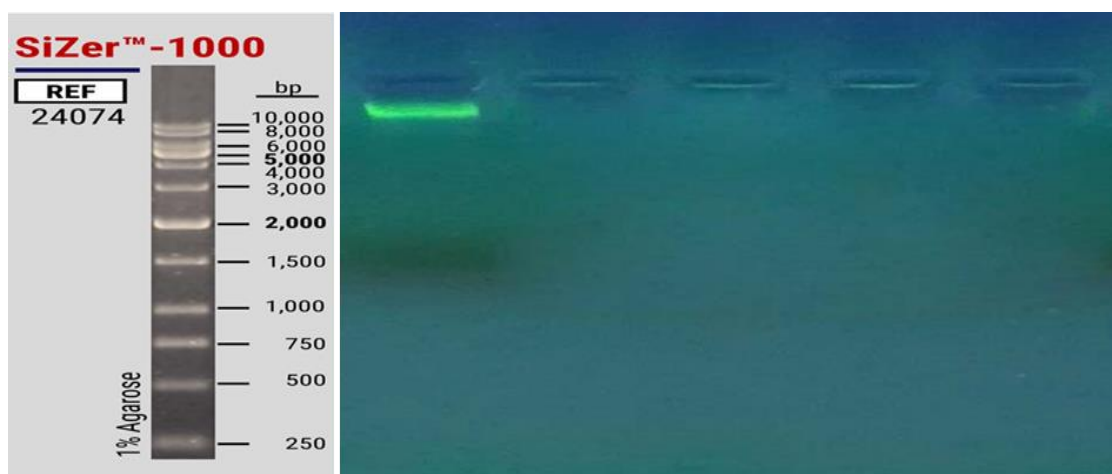

Fig. 5. Gel electrophoresis of genomic DNA extraction from Fungi *Rhizoctonia solani*, 1% agarose gel at 5 volt cm^{-1} for 1 hour; N: DNA ladder (1000).

PCR

Specific PCR results for the pair of specialized primers were observed on a gel electrophoresis device using 2% agarose gel, and the determination of the bands using an ultraviolet device and an illumination, UVT at a wavelength of 365 nm for the purpose. The results of the examination revealed the appearance of bands with size of 550 Pb (base pair), which is the expected size produced by the pair of specialized primers, and this band belongs to the fungus *R. solani* (Fig. 6).

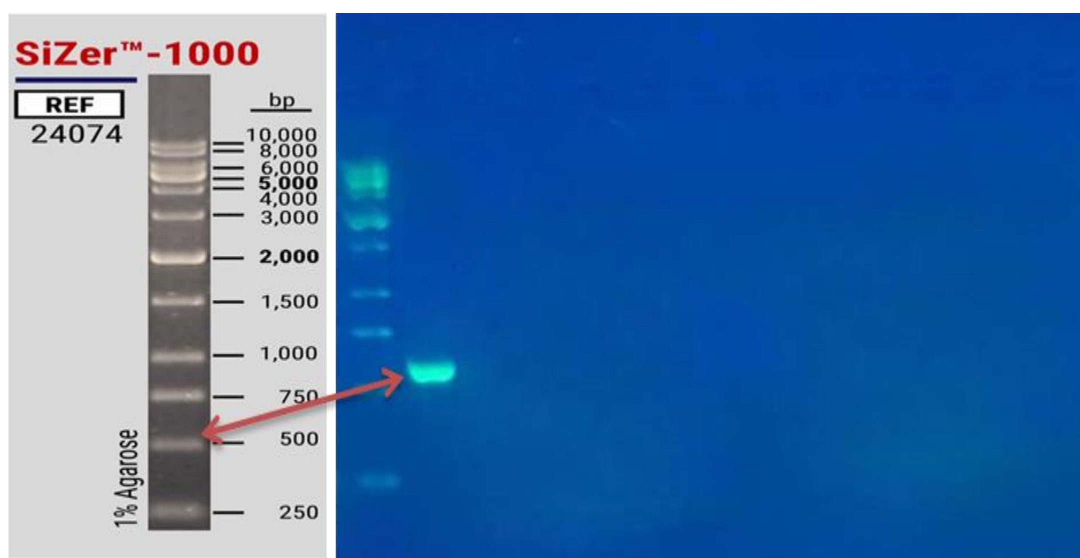

Fig. 6. PCR product showing band sizeof 550 bp. The product was electrophoresis on 2% agarose at 5 volt cm^{-2}; 1x TBE buffer for 1:30 hours; N: DNA ladder (1000).

Analysis sequencing

Depending on the results of nucleotide sequencing analysis, and comparing the results with the data of the same fungus available in the NCBI database, the pathogenic fungus, *R. solani* was first recorded as a cause of Aloe vera root and crown rot disease in NCBI under accession No. ID: MW498394.1 (Table 6 and Fig. 7). The results (Table 7) also showed that the compatibility rate ranged between 92-99% between the isolates under study and the isolates found in the gene bank. The *R. solani* isolate under study was 99% compatible with ID: MW498394.1 Iraq, ID: KX583263.1 Mexico, ID: JF701743.1 India and others, while 92% was identical to ID: HQ248215.1 Colombia and ID: KC625540. .1 Cuba, ID: AY684922.1 Taiwan. Fig. 8 also exhibits the genetic tree (cluster curve) dendrogram and the percentage of congruence between the isolates of *R. solani* recorded in the database and their convergence with the studied isolates. It was found that the percentage of genetic congruence between the isolate under study and the two isolates (9 and 10) is 97%, while the two other isolates (19 and 20) are 92%.

The results were also confirmed by matching ratio according to nucleotides sequence (nitrogenous bases) between *R. solani* isolated from Aloe vera and isolates recorded in the NCBI (Fig. 9). The results of the present study are in

agreement with those of Han *et al.* (2013) in the possibility of identifying the pathogen based on a short sequence of DNA strand within the nucleotide sequencing technique.

Table 6. Conformism ratio amongst copies particularly diagnosed and others copies at NCBI.

Score	Expect	Identities	Gaps	Strand
1122 bits (1243)	0.0	623/624 (99%)	0/624 (0%)	Plus/Plus

Query 1 GAGTTGGTTGTAGCTGGCCTAATAAATTAATGTTGGGCATGTGCACACCTTCTCTTTCAT 60
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 57 GAGTTGGTTGTAGCTGGCCTAATAAATTAATGTTGGGCATGTGCACACCTTCTCTTTCAT 116

Query 61 CCACACACACCTGTGCACCTGTGAGACAGTAGGGGATTTTTAATTTAATTTAATTGGACC 120
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 117 CCACACACACCTGTGCACCTGTGAGACAGTAGGGGATTTTTAATTTAATTTAATTGGACC 176

Query 12 CTCTGTCTACTTAATTCATATAAAATCAATTTAATTAAAAATGAATGTAATTGATGTAAC 180
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 177 CTCTGTCTACTTAATTCATATAAAATCAATTTAATTAAAAATGAATGTAATTGATGTAAC 236

Query 181 GCATCTAATACTAAGTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAG 240
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 237 GCATCTAATACTAAGTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAG 296

Query 241 CGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCA
300
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 297 CGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCA 356

Query 301 CCTTGCGCTCCTTGGTATTCCTTGGAGCATGCCTGTTTGAGTATCATGAAATCTTCAAAG 360
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 357 CCTTGCGCTCCTTGGTATTCCTTGGAGCATGCCTGTTTGAGTATCATGAAATCTTCAAAG 416

Query 361 TAAATCTTTTGTTAATTCAATTGGTTCTGCTTTGGTATTGGAGGTTATTGCAGCTTCACA 420
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 417 TAAATCTTTTGTTAATTCAATTGGTTCTGCTTTGGTATTGGAGGTTATTGCAGCTTCACA 476

Query 421 ACTGCTCCTCTTTGTGCATTAGCTGGATCTCAGTGTTATGCTTGGTTCCACTCAGCGTGA 480
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 477 CCTGCTCCTCTTTGTGCATTAGCTGGATCTCAGTGTTATGCTTGGTTCCACTCAGCGTGA 536

Query 481 TAAGTATCTATCGCTGAGGACACTGTAACAGGTGGCCAAGGTAAATGCAGATGAACCGCT
540
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 537 TAAGTATCTATCGCTGAGGACACTGTAACAGGTGGCCAAGGTAAATGCAGATGAACCGCT 596

Query 541 TCTAATAGTCCATTAATTTGGACAATATTTTTATGATCTGATCTCAAATCAGGTAGGACT 600
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 597 TCTAATAGTCCATTAATTTGGACAATATTTTTATGATCTGATCTCAAATCAGGTAGGACT 656

Query 601 ACCCGCTGAACTTAAGCATATCAT 624
||||||||||||||||||||||||
Sbjct 657 ACCCGCTGAACTTAAGCATATCAT 680

Fig. 7. Sense flanking of partial ITS gene sequencing in comparison with the gene MW498394.1 standard from Gene Bank; Here, the sample query signifies; Subject signify NCBI database.

Table 7. Compatibility rates between the *R. solani* isolate under study and other isolates found in the NCBI.

	Accession	Country	Source	Compatibility
1.	ID: MW498394.1	Iraq: Basrah	*Rhizoctonia solani*	99%
2.	ID: KX583263.1	Mexico	*Rhizoctonia solani*	99%
3.	ID: JF701743.1	India	*Rhizoctonia solani*	99%
4.	ID: MH025376.1	Oman	*Rhizoctonia solani*	99%
5.	ID: JX454675.1	India: Karnataka	*Rhizoctonia solani*	99%
6.	ID: LN735556.1	Egypt:Menufia	*Rhizoctonia solani*	99%
7.	ID: KX523896.1	Iran	*Rhizoctonia solani*	99%
8.	ID: JF701718.1	India: Delhi	*Rhizoctonia solani*	98%
9.	ID: MH014991.1	China	*Rhizoctonia solani*	97%
10	ID: JQ343829.2	China: Taian	*Rhizoctonia solani*	96%
11	ID: MH861382.1	Japan	*Rhizoctonia solani*	95%
12	ID: JF519844.1	Italy: South_Tyrol	*Rhizoctonia solani*	95%
13	ID: KT362072.1	Egypt	*Rhizoctonia solani*	95%
14	ID: AF153784.1	Australia: Sydney	*Rhizoctonia solani*	95%
15	ID: MG844390.1	Pakistan	*Rhizoctonia solani*	94%
16	ID: KF907736.1	Belgium	*Rhizoctonia solani*	94%
17	ID: DQ356413.1	USA: Washington	*Rhizoctonia solani*	94%
18	ID: HQ248215.1	Colombia	*Rhizoctonia solani*	92%
19	ID: KC625540.1	Cuba	*Rhizoctonia solani*	92%
20	ID: AY684922.1	Taiwan	*Rhizoctonia solani*	92%

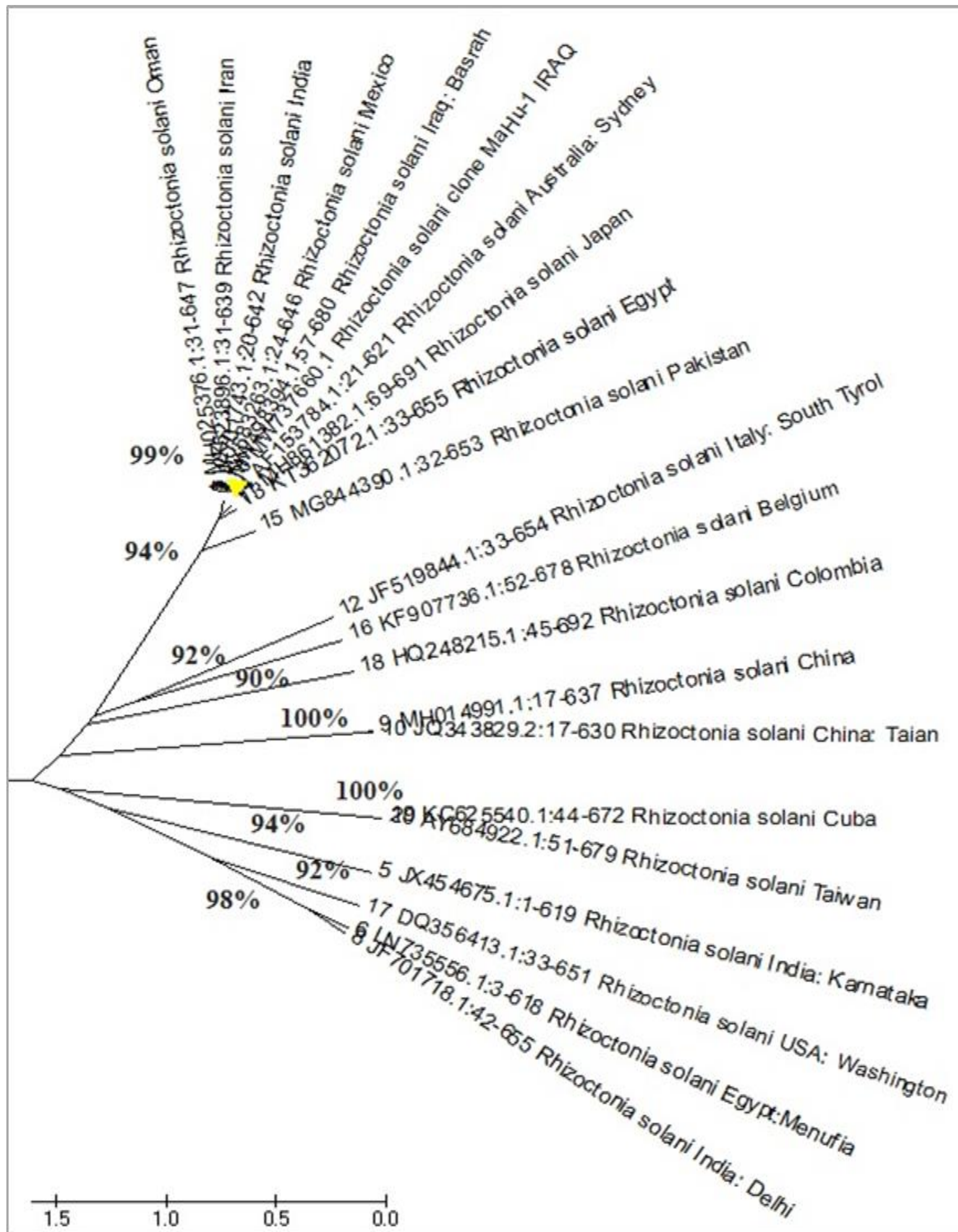

Fig. 8. The genetic tree (cluster curve) dendrogram showing percent relationship between *R. solani* under study and recorded isolates recorded in the NCBI database.

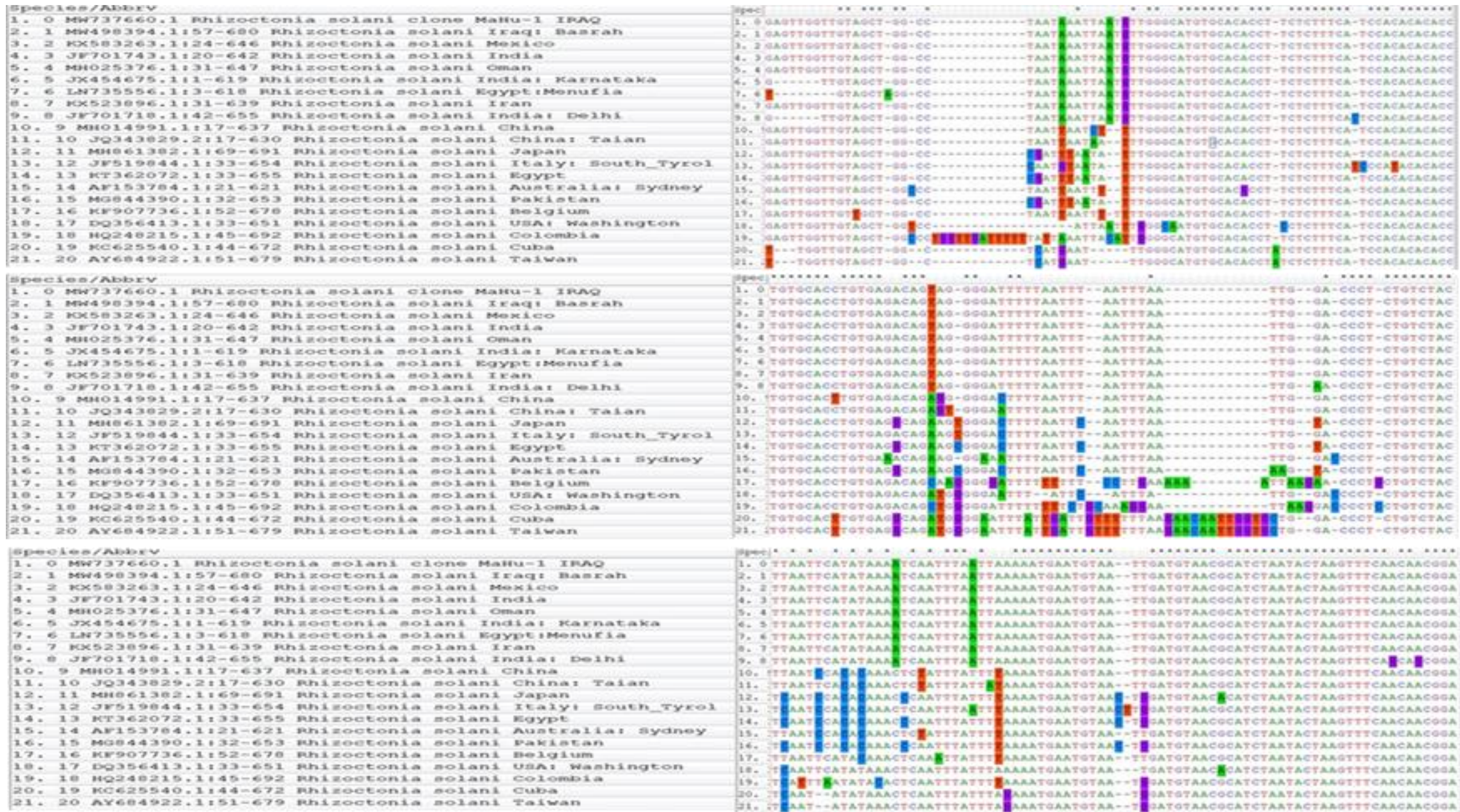

Fig. 9. Matching ratio according to nucleotides sequence (nitrogenous bases) between *Rhizoctonia solani* isolated from Aloe vera and isolates recorded in the NCBI database.

CONCLUSION

We conclude from this study that the symptoms of infection on the Aloe vera plant, which showed yellowing of the leaves, rotting of the roots and reddish-brown lesions, are caused by the pathogenic fungus *Rhizoctonia solani*. This is considered to be the first molecular record of this fungal pathogen in Iraq on Aloe vera plants.

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