Survey and Diagnosis etiology identification of root and crown rot disease of *Aloe vera* in Karbala, Iraq

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Abstract

During the growing season 2022-2023, root and crown rot disease symptoms were distributed epidemiologically on *Aloe vera* plants in the ornamental nurseries of Karbala Province, Iraq. Therefore, this study aimed to investigate this disease's etiology and survey its prevalence in Karbala Province, Iraq. Numerous ornamental nurseries were selected randomly to survey this disease and isolate, identify, and investigate the pathogenicity of the causative agent. The survey results showed this disease is prevalent in all the surveyed nurseries, with varying incidences between 28.14% and 80%. The isolation, morphological identification, and pathogenicity analysis revealed that the fungus causing this disease is *Rhizoctonia solani*. This identification was confirmed molecularly by nearly identical similarity (>99%) of the Internal Transcribed Spacer (ITS) sequence of this fungus with several international strains belonging to the same fungus. The root and crown rot disease of *A. vera* is widespread in the Karbala Province and may deteriorate in the coming years, causing economic losses. Therefore, further studies are needed to apply effective approaches to control it.

Keywords: *Aloe vera*, root and crown rot, Morphological and molecular identification , Rhizoctonia solani.

Introduction

Ornamental plants have multiple benefits, including recreational benefits, represented by providing psychological comfort to humans by creating an environment of pleasure and comfort, and environmental benefits, as they contribute to reducing environmental pollution by absorbing toxic gases [1,2]. The economic benefits are represented by contributing to raising the economic level of those working in ornamental plants through trade in flowers, indoor plants, and their production requirements [3, 4]. In addition, many ornamental plants have multiple medical uses, as in primary health care, to treat several human diseases [5, 6, 7]. One of these dual-use plants is the *Aloe vera* plant, which belongs to the *Asphodelaceae* family [8]. It is an evergreen perennial succulent plant in tropical and subtropical climates. It is believed to originate in the Arabian Peninsula region. However, it grows widely worldwide and is considered an invasive species in many regions of the world [9]. It is grown for ornamental purposes in the interior as a potted plant and mainly as a medicinal plant for topical treatment, as it has been used for centuries against many diseases, especially skin diseases, as *A. vera* leaves contain large quantities of acemannan gel polysaccharide, which can be used for a wide range of medical purposes, including skin lotions, cosmetics, ointments, and gels for minor burns [10, 11]. Thus, it is currently cultivated widely in many provinces of Iraq.

Rhizoctonia solani, a soil-borne necrotroph that is present universally, causes damage to a vast array of economically significant crops [12]. The members of this species exhibit a considerable range of diversity concerning their colony morphology, biochemical and molecular markers, pathogenicity, and aggressiveness. This diversity has allowed them to be categorized into fourteen anastomosis groups (AGs). However, these groups are somatically incompatible with each other [13]. It is a versatile fungus that causes a wide range of diseases in plants. It causes root rot, damping off, and wire stem, affecting a wide host range with a worldwide distribution [14]. This pathogen can also infect a wide array of vegetables and economic crops, causing root and/or crown rots, stem cankers, and leaf infections [15]. Moreover, it can cause seed decay, post- and pre-emergent damping-off, fruit decay, and foliar blights [16].

There are many nurseries in Karbala province, which is considered one of the main regions for the cultivation and production of this plant. These nurseries are affected annually by various plant pathogens, causing regular economic loss [17, 18, 19). In the 2022-2023 season, crown and root rot symptoms were observed severely and commonly in most of the nurseries of Karbala province. Thus, this study was conducted to determine the etiology of the disease and survey its distribution.

Materials and Methods

Survey, isolation, and morphological identification of the pathogen

A survey of *Aloe vera* plants was conducted from 1-11-2022 to 1-4-2023 in many nurseries (17 nurseries) selected randomly and spread in different areas of Karbala province. Root and crown rot disease incidence was estimated based on the symptomatic plants. The prominent disease symptoms were wilting and reddish-brown leaves in color, starting from the top to downwards. Then, these tips dry out, extending from the leaves' tips to their bases, accompanied by discoloration of the root system and crown in a reddish-brown color that turns into a dark brown color accompanied by rotting and softness. (Fig. 1).

Several symptomatic samples of leaves and roots of *A. vera* plants were collected randomly during the survey. These samples were washed well with distilled water and dried with filter paper [20]. After removing the dead parts, the tissues were cut into small pieces 1-5.1 cm long before performing the surface sterilization with a sodium hypochlorite solution of concentration 2. % prepared from the commercial solution for three minutes. The pieces were washed with sterile distilled water for two minutes three consecutive times and then dried with clean filter paper [21]. Each of the five disinfected pieces was transferred to a 9 cm diameter petri dish containing a medium of water agar that was prepared according to the instructions of the producing company (Indian Bioscience CDH) and sterilized. All plates were then incubated at 25 ± 2 °C in darkness, and the fungal growth was monitored daily [22]. The fungal isolates were purified by transferring a disk (5mm in diameter) from the tip of each colony grown and planting in a petri dish containing Potato Dextrose Agar (PDA) medium prepared according to the instructions of the producing company (Indian Bioscience CDH) and sterilized with an autoclave under the same conditions mentioned above and amended with the antibiotic Amoxicillin (100 mg/L) [23, 24, 25].

The pure fungal isolates were identified based on the morphological characteristics of the colony's growth and microscopic examination [26, 27]. The fungi were identified initially based on previously described taxonomic traits [28].

Figure (1): Symptoms of root and crown rot disease on *A. vera*

Molecular identification of the pathogen

The total genomic DNA was extracted from the pure fungal isolates using the DNeasy Plant Kit (Qiagen, Germany) with minor modification [12] and then used as a template in a standard Polymerase Chain Reaction (PCR). The Ready-To-Go PCR Beads kit (GE Healthcare, UK) was utilized for conducting the PCR reaction by preparing a final volume of 25 µL containing the main reaction components, including 1 µL of the ITS1 and ITS4 primers described below, which target the internal transcribed spacer (ITS) region in addition to 2 µL of total DNA.

Forward primer ITS1
5TCCGTAGGTGAACCTGCGG3`

Reverse Primer ITS4
5TCCTCCGCTTATTGATATGC3`

The PCR program begins with the initial denaturation step, for 5 minutes at a temperature of 95 °C, then 35 cycles consisting of three steps: denaturation for 40 seconds at a temperature of 95 °C, then annealing for 40 seconds at a temperature of 55 °C, then extension for a minute at a temperature of 72 °C. Then, the final extension was for 5 minutes at a temperature of 72 °C [29].

The PCR amplicons were sent to Macrogen Inc. in South Korea to determine the sequences of the ITS genetic region for each fungal sample. The clean sequence was deposited into the GenBank database under the accession number OQ976986. Afterward, BLAST analysis of the fungal sequence demonstrated >99% identity with the global *Rhizoctonia solani* sequences. Phylogenetic analysis was executed by consuming MEGA 11 using the neighbor-joining method [30].

Pathogenicity assessment

The pathogenicity of the fungus accompanying the infected plant was evaluated. This assessment was carried out in the horticultural canopy of the Agriculture College/ University of Kerbala. The organic fertilizer (Compost) was sterilized using an autoclave under typical conditions for one hour and repeated the next day. The isolated fungus inoculum was prepared and added to the compost according to the method of Buttner et al., [31], which includes mixing the contents of five dishes of pure colonies of the pathogenic fungus with 1000 ml of distilled water using an electric mixer for two minutes, after which the inoculum was added as 15 ml to each pot containing a healthy *A. vera* plant. Other healthy *A. vera* plants were in uninoculated pots for comparison. All pots were then placed in the canopy, with daily monitoring for the appearance of disease symptoms.

Results and Discussion

Survey, isolation, and morphological identification of the pathogen

The survey results (Table 1) showed that the root and crown rot disease is distributed in all nurseries investigated, located in the main regions of Karbala Province. The disease incidence was confined between 18.1 to 80%. The nurseries of the Al-Ataishi area/ Al-Husseiniya region were the most affected by achieving the highest rate of disease incidence average, which reached 45.72 %. In contrast, the lowest rate was found in the nurseries of the Abbasid shrines, which amounted to 20.06 %.

Table 1. Root and crown rot disease incidence and severity in Karbala Province

Disease Severity Index (DSI) %	Disease incidence %	Area	Nursery name
65	22.2	Al-Husseiniya region	Al-Ataishi
72	50	Al-Husseiniya region	Brothers
90	80	Al-Husseiniya region	Al-Nour
78	58.3	Al-Husseiniya region	Al-Zahra
45	18.1	Al-Husseiniya region	Al-Husseiniya Agricultural
38	14.28	Al-Moallem region	Al-Mojtaba
55	23.4	Al-Moallem region	Raed Badri
60	33.7	Al-Moallem region	Fouad
70	46.66	Al-Moallem region	Karbala spring
72	46.85	Al-Moallem region	Nursery Al-Safa
42	18.18	Al-Moallem region	Al-Issa
68	40.77	Al-Moallem region	Moonlight
70	43.7	Al-Moallem region	Every Green
58	32.5	Al-Moallem region	Abu Anmar
50	20.06	Al-Mashatel region	Al-Abbasid shrine
60	30.03	Al-Hafez region	Al-Hussainiya Shrine

The isolation and morphological identification results revealed the presence of *R. solani* fungus in all infected plants. It was identified based on its growth culture and the asexual characteristics, represented by the formation of colonies in white color at the beginning of its growth that turn light to dark brown color in its late stage of growth (Fig. 2A) beside produce divided mycelia with perforated septa (Dolipore septum) that allows for movement of cytoplasmic contents. Also, it produces a typical right-angled branching with a septum located near the point of origin of branching. There is an apparent constriction at the branching point (Fig.2 B). Cells in mature hyphae contain brown pigmentation. Any conidia was not observed. However, the fungus usually begins to produce sclerotia that are irregular, cohesive, and solid brown to black clumps of dead and living hyphae that are hardy and resistant to harsh environmental conditions with an average length of 3 to 5 mm [16, 32].

Table (2): Frequency of fungi isolated from the infected samples (NC5)

Frequency of Occurrence (%)	No. of Isolates	Fungal Genus/ Species
66.2	43	*Rhizoctonia solani*
15.1	14	*Fusarium oxysporum*
10.4	7	*Pythium* spp.
8.3	6	*Alternaria* spp.
100%	70	Total

Figure (2): The morphological cultural and microscopic characteristics of the *R. solani* fungus accompanying the infected *A. vera* plants; (A) the upper surface of the fungal culture on PDA medium; (B) the microscopic features of the fungus.

Molecular identification of the pathogen

To confirm the morphological identification of *R. solani* fungus associated with the diseased *A. vera* plants, the most pathogenic
PCR amplification of DNA extracted from R. solani produced a DNA product of approximately 600 bp (Fig. 3).

The isolate (e.g., RS-K1) obtained from Al-Husseiniya region showed the highest pathogenicity level, causing 100% plant death within 10 days of inoculation. isolates were selected to determine molecular identification.

The molecular results confirmed the fungus identity by the high similarity (99-100%) of its ITS-rDNA sequence with numerous global isolates of *R. solani* (Fig.4). Thus, the isolate of this fungus was named *Rhizoctonia solani* isolate Karbala/Alovera-1 and deposited at the Genbank database/ NCBI under accession number OQ976986.

Results of the phylogenetic analysis confirmed this identification by gathering the same fungal isolate into a group clade consisting of several international isolates belonging to the *R. solani* fungus (Fig. 5).

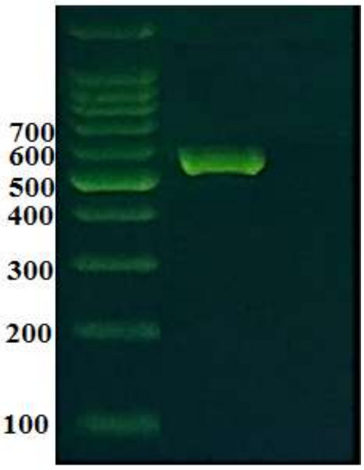

Figure (3): Agarose gel electrophoresis showing the PCR-amplified products of *R. solani* isolate.

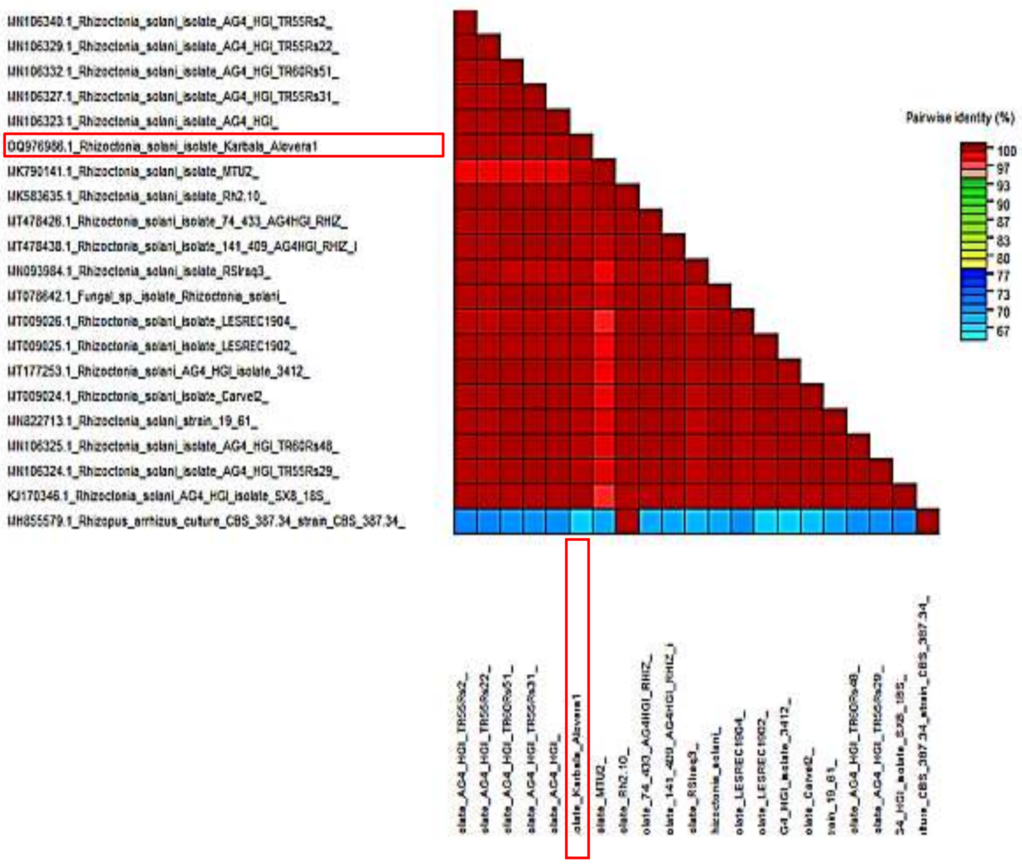

Figure (4): The similarity among the fungus *R. solani* isolate Karbala/Alovera-1 identified in this study (marked with a red frame) with the global isolates and strains of the same fungus, displaying a color-coded matrix showing the degree of similarity.

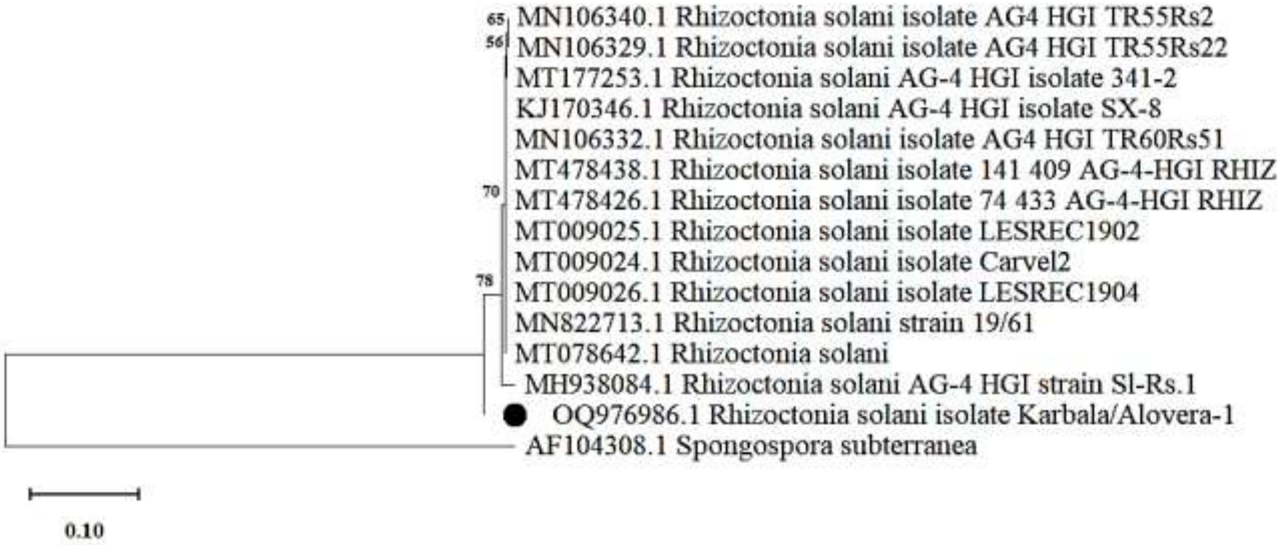

Figure (5): The phylogenetic relationship among the fungus *R. solani* isolate Karbala/Alovera-1 identified in this study (marked with a black dot) and other isolates and strains of the same fungus. The phylogenetic analysis was built based on the sequences of the rDNA-ITS region. The out-group was *Spongospora subterrena* (AF104308).

Pathogenicity assessment

The results of this assessment proved the pathogenicity of the fungus *R. solani* isolate Karbala/Alovera-1 isolated in this study by causing the typical disease symptoms of root and crown rot that appeared on all tested plants, with an infection rate of 100%. In contrast, no symptoms were noticed in the control plants (Figure 6).

Figure (6): The pathogenicity of *R. solani* isolate Karbala/Alovera-1on *A. vera* plants; (A) Infected plant (B) healthy plant.

The etiology of roots and crown rot disease of *A. vera* was identified based on its morphological, molecular, and pathogenicity features. It was pervasive in all nurseries of Karbala Province.

R. solani is one of the most important worldwide soil-borne pathogens that cause various diseases, including rots of seeds, roots, and crowns of many plant hosts [33, 34]. This is due to its successful parasitic process that depends on secreting various hydrolytic enzymes such as cellulase, chitinase, protease, and pectinase, enabling it to decompose cell walls and penetrate multiple plant tissues. Furthermore, it secretes some toxic compounds, such as oxalic acid, which kills cells to extract nutrients from them [35, 36].

This result is consistent with a previous study, which indicated the ability of *R. solani* fungus to infect *A. vera* plants in the nurseries of Nineveh Province, Iraq. In this study, Al-Ethawi and Al-Taae (37) found that the fungus *R. solani* had the highest frequency and was the leading cause of root and crown rot disease in *A. vera* plants. It is worth mentioning that the Nineveh Province is located in the north of Iraq, while the Karabal Province is in the middle of Iraq, which means this pathogen is

This indicates the widespread of this pathogen in *A. vera* plants, with the possibility of presenting in other provinces of Iraq. However, further studies are required to prove this fact, and additional efforts are urgent to find the best and alternative approaches to combating it and reducing its economic damage [Lahuf et al., 2020b].

Biological Control of *Rhizoctonia solani* Using *Trichoderma harzianum*

The current study identified the pathogen *Rhizoctonia solani*, which infiltrated the substrate of *Aloe vera*, as causing the root and crown rot disease in *Aloe vera* due to its extensive distribution and virulence. Thus, there is a need for sustainable methods for managing soil borne diseases caused by *R. solani* in nurseries. The evaluation of *T. harzianum* as a possible biocontrol agent for *R. solani*, and integration of *T. harzianum* with other components of integrated disease management, were investigated.

Results from an in vitro dual culture assay (DCA) showed that *T. harzianum* acted as an antagonist to *R. solani* by inhibiting the radial growth of the *R. solani* strain due to competition and direct mycoparasitism, two mechanisms of *Trichoderma spp*. known to occur for thousands of years (Gams&Bissett, 1998).

In greenhouse experiments, application of *T. harzianum* in soils reduced the severity of root and crown rot in plants when compared with the severity in plants inoculated with *R. solani*. In addition, plants that were treated with *T. harzianum* showed enhanced plant vigor, which indicates that *T. harzianum* has the ability to suppress *R. solani* in semi-controlled environments.

Table (3): Effect of *Trichoderma harzianum* on the severity of root and crown rot of *Aloe vera* caused by *Rhizoctonia solani*.

Disease reduction (%)	**Disease severity (%)**	**Treatment description**	**Treatment code**
0.0	85.0	Plants inoculated with *Rhizoctonia solani* only	**T1**
94.1	5.0	Plants treated with *Trichoderma harzianum* only	**T2**
70.6	25.0	Plants inoculated with *R. solani* + treated with *T. harzianum*	**T3**
100	0.0	Control (non-inoculated plants)	**T4**

Values represent mean disease severity evaluated 21 days after inoculation. Disease reduction was calculated relative to the pathogen-only treatment (T1).

Many articles support previous research that showed using *Trichoderma spp*. to control *R. solani* occurs because *Trichoderma spp*. lowers the density of pathogen inoculum and improves plant health (Wilson et al., 2008; Parajuli et al., 2025). The effect of *T. harzianum* on pathogen suppression operates through several biological mechanisms, including mycoparasitism, nutrient & space competition, and the production of cell wall degrading enzymes (chitinase and beta-1,3-glucanase), and through inducing systemic acquired resistance in host plants (Taheri & Tarighi, 2012). Pathogen establishment and disease progress are inhibited by the combined effects of these biological mechanisms occurring within the rhizosphere. As such, *T. harzianum* can be utilized as an environmentally sustainable and effective option for implementing chemical control strategies as part of the disease management program for *Aloe vera* root & crown rot. Implementation of this method supports current trends in sustainable agriculture and integration of soil-borne plant disease management strategies (Agrios, 2005; Akber et al., 2023).

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