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Full Length Article

First Morphological and Molecular Identification of Crown and Root Rot Pathogens of the Ornamental Plants, *Gazania* spp. and *Amaranthus cruenthus* in Iraq

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Abstract

During 2020–2021, Nursery-grown *Gazania* spp. and *Amaranthus cruentus* displayed severe crown and root rot symptoms, commonly observed in Babylon and Karbala Provinces of Iraq nurseries. The causative agents associated with these symptomatic crown and root tissues were the fungus *Fusarium oxysporum* on *Gazania* spp. and *F. oxysporum* and *Lasiodiplodia theobromae* on *A. cruentus.* This identification has relied on the pathogen's morphological, molecular, and pathogenic features. The combination of morphological and molecular characteristics was applied, incorporating the sequence data of the internal transcribed spacers (ITS) and small subunit (SSU) markers. In pathogenicity assessments, the disease symptoms were observed on the tested plants after one month, and the pathogens were re-isolated and identified, fulfilling Koch's postulates. This is the first report of crown and root rot diseases caused by *F. oxysporum* on *Gazania* spp. and *F. oxysporum* and *L. theobromae* on *A. cruentus* in Iraq. © 2024 Friends Science Publishers

Keywords: Crown and root rot; *Fusarium oxysporum*; *Lasiodiplodia theobromae*; Morphological and phylogenetic analysis; Ornamental plants

Introduction

Gazania (Asteraceae family) is a genus of important ornamental plants worldwide. The origin of this genus is supposed to be in South Africa (Raimondo *et al.* 2009). It is a stemless perennial 20–25 cm long with woody branches. The leaves are gathered at the tips of the branches and divided into the middle part with different shapes, from oval to very narrow linear inverted spears. The underside of the leaves is white. The flowers are characterized by different colors, including yellow, red, and orange, and the width of the heads of flowers is 35–65 mm with a dark ring inside the head. The protrusions surrounding the flower head are coarsely hairy (Goldblatt and Manning 2000; Magee *et al.* 2011).

Amaranthus cruentus belongs to the Amaranthaceae family and is commonly known by several names, such as the lover's blood, blood leaves, and herbal blood leaf. It may be native to tropical South America, specifically Brazil. It is also present in Asia's tropical regions and several parts of India (Feo 2003). Many researchers have identified multiple medical benefits of this plant, including its use in treating fever and kidney disorders. Furthermore, it has anti-allergic, anti-cancer, anti-inflammatory, antipyretic, and antioxidant capabilities (Schmidt *et al.* 2009). Moreover, *A. cruentus* is characterized by the beauty of its leaves and red colour, so it is an essential ornamental plant in Iraq and worldwide (Dakheel 2020).

The crown and root rot disease is considered one of the most important diseases affecting many plant hosts (Gonzalez *et al.* 2011; Nzungize *et al.* 2011; Lahuf *et al.* 2019a, 2022a, b). The symptoms of this disease initiate subterraneously and are initially indiscernible. However, in the later phase, they become noticeable on the upper part of the infected plant. This can lead to significant damage, sometimes uncontrollable (Hamon *et al.* 2011). Generally, the most prominent symptom associated with this disease is the transformation of the crown and root color to brown with softening as they become tender and decomposing, accompanied by the formation of blotches or spots on the tissues of the crown and roots from dark reddish to dark brown color, with the root split. These disease symptoms accompany leaves yellowing, wilting, plant growth and low yield (Irulappan and Senthil-Kumar 2021). This disease is caused by various pathogenic microorganisms such as bacteria and viruses (Legg *et al*. 2011) and fungi (Abdulmoohsin *et al*. 2019). Fungal pathogens are the most abundant causative agents of crown and root rot, which can rest during the winter season in the host remains, weeds, and infested soils for several years (Berg *et al*. 2017; Khan and

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Javaid 2022). Several members of the *Fusarium* genus have caused rot and other root diseases in numerous plant hosts (Akhtar *et al*. 2020; Naqvi *et al.* 2022). For example, *Fusarium solani* and *F. avenaceum* were the leading causes of this disease in leguminous crops (Lops *et al*. 2013) as they cause significant reductions in the production of beans (Cohen *et al*. 2014) and lentils (Sweets and Wright 2008). The fungus *F. solani* also causes severe rotting of cassava roots (AbdAllah *et al.* 2011). Additionally, the crown and root rot on potato crop was observed (Alhamiri 2016). Furthermore, *F. oxysporum* was reported to cause this disease on yacon potatoes in Brazil (Moraes *et al.* 2017) and *Polygonatum sibiricum* in China (Li and Guo 2019). Additionally, *F. oxysporum* was identified as causing crown and root rot on Gazania reigns plants in Argentina (Wright *et al.* 2007). Furthermore, *Lasiodiplodia theobromae* (syn. *Botryodiplodia theobromae*) was found to cause black root rot, and *Macrophomina phaseolina* has caused charcoal root rot in India (Pinto *et al*. 2018). However, no such a report has been found regarding this disease on Iraq's *Gazania* spp. *or A. cruentus* plants. Thus, the current study was accomplished to identify and characterize the causal agents of crown and root rot disease on these two ornamental plants in Babylon and Karbala Provinces, Iraq.

Materials and Methods

Pathogens isolation

Numerous samples of *Gazania* spp. and *A. cruentus* showing severe crown and root rot symptoms were collected during a survey conducted in the growth season of 2020–2021 from several nurseries of Babylon and Karbala Provinces/Iraq. The disease symptoms were dark brown color associated with the softness of the infected tissues extending to the Crown region. Subsequently, the diseased plants wilted, rotted and died. The diseased tissues of the crown and roots were cut into small segments (1–2 cm long), washed and surface-disinfected using 1% (w/v) of sodium hypochlorite. The disinfected tissues were dried and spread on water agar media as five segments per plate. All the plates were then incubated for three days at 25°C (Lahuf *et al*. 2020). The hyphal tip technique was applied on all emerging colonies by relocating each colony tip into potato dextrose agar (PDA) media amended with the antibiotic amoxicillin (200 μg mL^{-1}). Consequently, the inoculated plates were incubated at 25°C for a week (Hwang *et al.* 2017; Hameed *et al.* 2021).

Morphological identification

The morphological features of the 7-day-old pure colonies grown on PDA medium and incubated at 25°C in darkness were examined. Cultural appearances were monitored, including mycelial structure, color and growth obtained. Conidial morphologies of the pure colonies were verified, recorded and compared with previous descriptions (Leslie and Summerell 2006; Gnanesh *et al*. 2022).

Pathogenicity examination

To assess the pathogenicity of these fungal isolates, healthy 4-week-old Gazania spp. and A. cruentus seedlings were transplanted into autoclaved and inoculated soil. The inoculation was made based on Dewan protocol (1989), which was prepared using seeds of local millet (*Panicum miliacem*) for the loading purpose of the selected fungus. The seeds were first washed with water to remove the dust and impurities debris; then they were soaked for six hours, placed on a piece of gauze to eliminate the excess water and distributed with equal weights in flasks (250 g per flask) and autoclaved for 20 min. After the sterilization process, five discs (with a diameter of 0.5 cm) were taken from the pure colonies of the isolated fungi used to inoculate the autoclaved millet seeds. All the inoculated flasks were incubated at 25 ± 2°C for 14 days, considering the monitoring or follow-up every two days. The content was shaken to ensure the distribution of the fungal inoculation on all seeds equally. Subsequently, the sterilized soil was inoculated by adding 10% of the fungal inoculation and mixed with the soil well to ensure homogenization. The combination was incubated at 25°C for three days and spread into plastic pots. One seedling was transferred to each pot. Furthermore, healthy seedlings were planted in sterile soil for compression. Afterward, all inoculated and control pots were incubated in a plastic house (Lahuf *et al.* 2018a). Irrigating of the seedlings was implemented as needed for 60 days. The fungal pathogens were re-isolated from plants showing the disease symptoms. The morphological appearances of the re-isolated fungi were compared with the analogous isolates to complete the requirement of Koch`s postulates. However, all control seedlings were symptomless (Lahuf *et al.* 2020).

Molecular identification

The DNeasy Plant Mini kit (Qiagen, Germany) was used to extract whole genomic DNA of 7-day pure fungal isolates (Lahuf *et al.* 2019b). The polymerase chain reaction (PCR) test was operated with the universal primer pair (ITS1/ITS4) to amplify the Internal Transcribed Spacers (ITS-rDNA) (White *et al*. 1990; Lahuf 2019a, b). In addition, specific primer sets (NS1/ NS8) were utilized to amplify the small subunit of the ribosomal ribonucleic acid (SSU-rDNA) (Raja *et al.* 2017).

The PCR products were sequenced at Macrogen Inc. in Seoul/South Korea. The nucleotide sequences of the genetic markers obtained were analyzed *via* Chromas software version 2.6.4. Afterwards, Basic Local Alignment Search Tool (BLAST) analysis was run to compare the edited sequences with the database of the GenBank at the National Center for Biotechnology Information National

(NCBI). Subsequently, the Molecular Evolutionary Genetics Analysis (MEGA) Version 10.1.5 was employed for phylogenetic analysis sequence data and operational the Neighbour-joining method of the ITS-rDNA and SSU-rDNA sequences of the current study and others documented in the NCBI-GenBank to build phylogenetic tree (Tamura *et al*. 2013; Lahuf *et al*. 2018b). Edited sequences of the fungi isolated in this study were deposited to the NCBI-GenBank Database.

Results

Pathogen isolation and morphological identification

Fungi were consistently obtained from the diseased crown and root tissues of *Gazania* spp. and *A. cruentus*. The colony growth was quick, forming radial aerial mycelia that are white, which later turn into a light or dark pink color, in addition to the different colors of the pigments secreted in the culture medium (Fig. 1A and 2A). The fungal colonies produced two types of conidia: macroconidia, which were distinctly curved falciform and spindle-like-shaped with thick-wall; most had 3–5 septate. On the other hand, the microconidia were kidney to broad ovate and elliptic-like-shaped with 0–1 septate (Fig. 1B and 2B). The chlamydospores were rare, single or in clusters and were terminal or intercalary. These cultural and microscopic features indicate that the fungus is a member of the *Fusarium* genus.

Additionally, another fungus was constantly isolated from the same diseased tissues of *A. cruentus* plants. It formatted an aerial fluffy rounded to irregular boundary colony that were initially greyish white colour that turned to dark grey later. It has grown radially equally and coated the surface of the media within five days (Fig. 3A). Abundant dark pycnidia structures were observed after four weeks. The immature conidia were translucent, elliptical, and thick-walled. After maturity, they become dark brown to black with one transverse septum (Fig. 3B). Dark liquid exudations were produced on the surface of colonies. These cultural and microscopic traits denoted that the fungus is a species of the *Lasiodiplodia* genus.

Pathogenicity examination

All inoculated *Gazania* spp. and *A. cruentus* plants showed crown and root rot symptoms within three weeks of inoculation. However, the non-inoculated plants did not display any disease symptoms. The causal agent, *F. oxysporum* and *L. theobromae* were re-isolated separately from all inoculated plants, and their microscopic characters were comparable to those of the original isolates. Accordingly, Koch`s postulates were fulfilled successfully, and it uncovered that *F. oxysporum* was the pathogen of the crown root rot disease on *Gazania* spp. (Fig. 4). The same fungus was pathogenic to *A. cruentus* plant that was also infected by another fungus *L. theobromae* (Fig. 5).

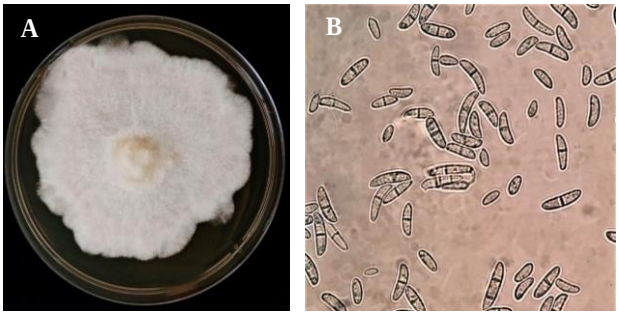

Fig. 1: Cultural and microscopic morphological characteristics of *F. oxysporum* isolated from the diseased crown and root of *Gazania* spp. (**A**) Colony of *F. oxysporum* grown on PDA after seven days; **(B)** Macro- and microconidia of *F. oxysporum*

Fig. 2: Cultural and microscopic morphological appearances of *F. oxysporum* isolated from the diseased crown and root of *A. cruentus* **(A)** Colony of *F. oxysporum* grown on PDA after seven days; **(B)** Mycelium, macro- and microconidia of *F. oxysporum*

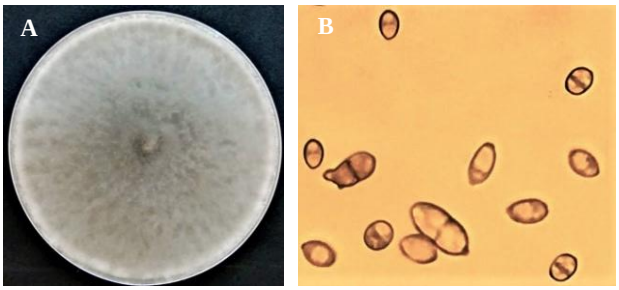

Fig. 3: Cultural and microscopic morphological features of *L. theobromae* isolated from the diseased crown and root of *A. cruentus*. **(A)** Colony of *L. theobromae* grown on PDA after seven days; **(B)** Conidial morphological characteristics of *L. theobromae*

Molecular identification

The PCR products of the ITS and SSU-rDNA regions amplified were 400–600 bp and 800 bp, respectively. BLAST analysis of this sequence demonstrated 99–100% similarity with those documented sequences of *F. oxysporum* and *L. theobromae* deposited in the GeneBank database. Thus, the sequences of the F. oxysporum and L. theobromae genetic markers. Isolates were deposited in the

Fig. 4: Crown and root rot symptoms emerged on the inoculated *Gazania* spp. **(A)** the inoculated Gazania plant with *F. oxysporum* strain F.oxy. ARF-3 while **(B)** Non inoculated plant

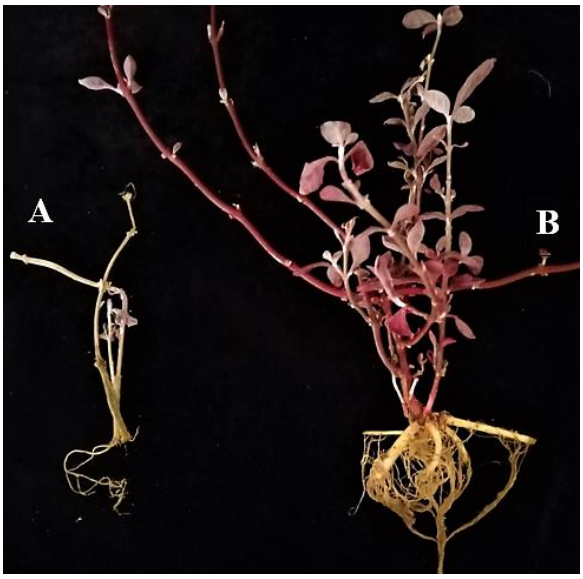

Fig. 5: Crown and root rot symptoms emerged on the inoculated *A. cruentus* **(A)** the inoculated *A. cruentus* plant with *F. oxysporum* strain. ARF-4, or *L. theobromae* ARF-1, whereas **(B)** Non-inoculated plant

NCBI GenBank under accession numbers MZ501950.1 and MZ501951.1 for the SSU marker sequence of *F. oxysporum* isolated from *Gazania* spp. plants. On the other hand, the

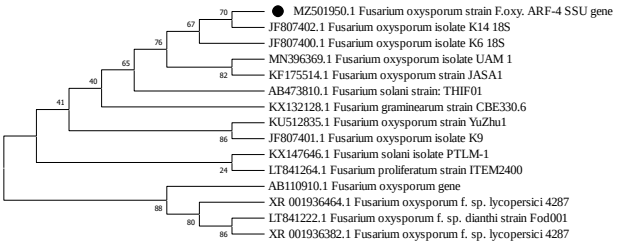

Fig. 6: Phylogenetic tree constructed using the SSU-rDNA sequences of the *F. oxysporum* obtained from the diseased *Gazania* plants in this study (determined by black dote) and other strains of the same species retrieved from GenBank-NCBI

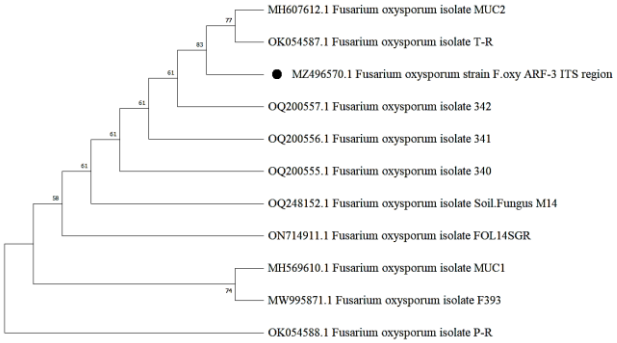

Fig. 7: Phylogenetic tree constructed using the ITS-rDNA sequences of the *F. oxysporum* attained from the diseased *A. cruentus* plants (determined by black dote) and other strains of the same species retrieved from GenBank-NCBI

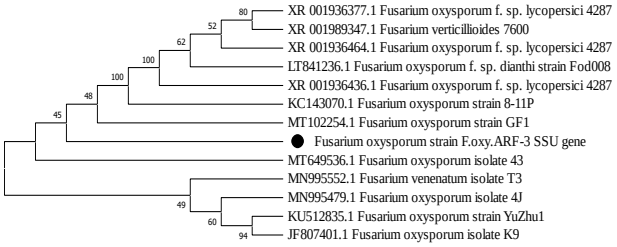

Fig. 8: Phylogenetic tree completed using the SSU-rDNA sequences of the *F. oxysporum* attained from the diseased *A. cruentus* plants (determined by black dote) and other strains of the same species retrieved from GenBank-NCBI

sequence of the SSU and ITS markers were MZ496571.1 and MZ496570.1 for *F. oxysporum*, besides MZ504270.1 and MZ496569.1 for *L. theobromae* isolated from *A. cruentus* plants.

The phylogenetic analysis demonstrated that the strains of *F. oxysporum* obtained in this study from *Gazania* spp. and *A. cruentus* were grouped with numerous global isolates of *F. oxysporum* (Fig. 6, 7 and 8). Similarly, the *L. theobromae* fungus was also grouped with numerous international reference isolates of the same fungus (Fig. 9 and 10).

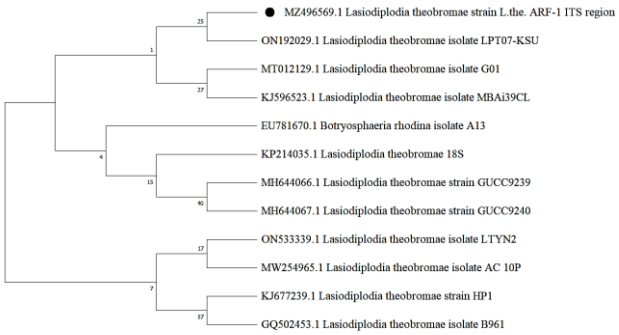

Fig. 9: Phylogenetic tree constructed using the ITS-rDNA sequences of the *L. theobromae* collected from the diseased *A. cruentus* plants (determined by black dote) and other strains of the same species retrieved from GenBank-NCBI

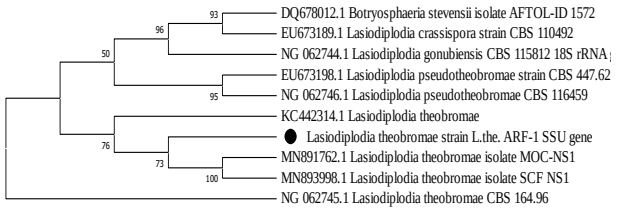

Fig. 10: Phylogenetic tree constructed using the SSU-rDNA sequences of the *L. theobromae* collected from the diseased *A. cruentus* plants (determined by black dote) and other strains of the same species retrieved from GenBank-NCBI

Discussion

The present investigation indicated that crown and root rot disease is common in nurseries of Babylon and Kerbala Provinces, Iraq. The fungal pathogen of this disease on *Gazania* spp. and *A. cruentus* plants based on its morphological and molecular characterizations is *F. oxysporum* (Leslie and Summerell 2006; Li and Guo 2019). Additionally, *L. theobromae* fungus was identified to cause the same disease in *A. cruentus* plants.

Diseases on ornamental plants arise due to various pathogens, including *F. oxysporum*, either during the cultivation process or the storage of bulbs and corms. There are two distinct types of symptoms associated with the fungus, namely vascular wilt and crown and root rot (Brayford 1992). The fungus, *F. oxysporum*, infiltrates the roots of the host plant, gradually progressing towards the xylem vessels. The cortex layers are degraded by the fungus, resulting in the formation of severe necrotic spots that range in color from brown to black. Ultimately, this leads to basal plate rot (Baayen and Rijkenberg 1999). In light of this, one may question whether the symptoms, typically classified as rotting, could potentially be categorized as advanced wilting symptoms on these distinct organs. In fact, numerous studies reported *F. oxysporum* as a wide host-pathogen infecting various plants causing root rot disease, such as tomato (*Solanum lycopersicum*) (Halim 2001); cucumber (*Cucumis sativus*) (Komy *et al*. 2021); sweet pepper (*Capsicum annuum*) (Gilardi *et al.* 2019) and *Ophiopogon bodinieri* (Lu *et al.* 2020). Furthermore, *L. theobromae* was recorded also as the causative agent of root rot on various plant hosts such as Mulberry (Gnanesh *et al.* 2022). Molecular identification methods, particularly PCR, have presented an alternative strategy for the discovery and characterization of numerous soilborne pathogenic fungi (Jaber and Lahuf 2020). Among the most commonly investigated genetic markers is the ITS rDNA due to its specificity to certain species, which makes it a more desirable option for phylogenetic analyses within the pathogen species complex, as it offers improved resolution at the sub-species level. As such, sequence analysis emerges as a superior choice for these phylogenetic investigations (Shehan *et al.* 2022).

Although *F. oxysporum* and *L. theobromae* are phytopathogens that are predominantly globally infecting various plant hosts, they have not been previously described in Iraq infecting *Gazania* spp. and *A. cruentus* plants. Thus, this is the first report of *F. oxysporum* causing crown and root rot on *Gazania* spp. and *A. cruentus* plants in Iraq. The first report of *L. theobromae* induces crown and root rot on *A. cruentus* plants in Iraq.

Conclusion

In Iraq, pathogens identification of crown and root rot of ornamental plants has been scarcely exploited. The present findings provide evidence that the fungi *F. oxysporum* and *L. theobromae* are the causative agents of crown and root rot disease on the ornamental plants *Gazania* spp. and A. crunches for the first time in Iraq. This particular disease has been observed to be prevalent in the nurseries of two provinces in Iraq. As a result, it is imperative to conduct further investigations in order to explore the impact of these two pathogens on other types of ornamental plants. Additionally, it is crucial to evaluate and determine the most effective strategies to successfully manage and control the spread of these pathogens in order to safeguard the overall health and well-being of the ornamental plants.

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Author Contributions

A L planned the experiments, F D and B G did the survey and collected the samples, A L and F D interpreted the results, F D, A L and R A wrote the manuscript, and M A statistically analysed the data.

Conflict of Interest

The authors of the current investigation would like to declare that there is no conflict of interest.

Data Availability

The data related to this study can be found at NCBI GenBank under accession numbers: MZ501950.1, MZ501951.1, MZ496571.1, MZ496570.1, MZ504270.1 and MZ496569.1.

Ethics Approval

It is not related to the current study

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