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IDENTIFICATION AND CHARACTERIZATION OF FUNGAL PATHOGENS CONTRIBUTING TO CITRUS TWIG DIEBACK IN KERMAN PROVINCE, IRAN

Maryam Rouzbeh
Kerman Agricultural and Natural Resources Research Center, Kerman, Iran.

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ABSTRACT

Citrus decline with complex symptoms was recently observed in Kerman province, reducing the annual yield of citrus fruits. Agents that cause citrus decline include fungi, bacteria, viruses, nematodes, and abiotic factors. One of the most important symptoms of citrus decline is branch dieback. In this study, the fungal agent responsible for dieback was identified. To achieve this goal, sampling was conducted in citrus orchards with symptoms of twig dieback and twig canker in the summer of 2020. Samples were collected from different areas of the citrus growing region of Kerman province, including Bam, Rigan, Narmashir, Fahraj, Shahdad, and Orzueeh. Fungi were isolated from diseased branches on nutrient culture medium. The resulting fungi were identified using morphological characteristics. The following fungi were isolated from diseased plants: *Alternaria* spp., *Nattrassia mangiferae*, *Ulocladium* spp., *Bipolaris* spp., *Fusarium* spp., *Phoma* sp., *Rhizoctonia* sp., *Paecilomyces* spp., *Chaetomium* spp., *Acremonium* spp., *Stemphylium* sp., and *Cladosporium* spp. Notably, *Alternaria* spp. exhibited the highest frequency and widest distribution among them, making it the most prevalent fungus throughout all citrus-growing regions of Kerman province. *N. mangiferae* was the second most frequently isolated fungus. Pathogenicity tests were performed by artificially inoculating two-year-old lemon trees under controlled conditions. All fungal genera except *Chaetomium* sp. and *Cladosporium* spp. were virulent. *N. mangiferae* was the most virulent fungus and caused the most severe cankers six weeks after inoculation. It also had the greatest discoloration compared to other fungi in these tests.

Corresponding Author: Maryam Rouzbeh
Email: maryam.rouzbeh@gmail.com

INTRODUCTION

Kerman province, Iran, is one of the largest citrus producers in the country. It ranks eighth in terms of citrus output, producing 35,843 tons of citrus fruits every year (Anonymous, 2021). However, in recent years, citrus fruits have shown symptoms such as dieback, bark splitting, wilting, and decline, which are common problems in citrus groves. Citrus decline is a complex global problem that reduces the fertility and productivity of citrus trees. Various fungal agents have been identified as the causes of citrus dieback in different regions of the world. For example, in California, *Neocytalidium dimidiatum*, formerly known as *Hendersonula toruloidea*, was reported as the causal agent of citrus dieback in the 1950s (Mayorquin et al., 2013). Similarly, dieback and canker in Arizona citrus have undergone investigation, leading to the isolation of *Phytophthora nicotianae* and *P. citrophthora* (Olsen, 2000). In Venezuela, *Lasiodiplodia theobromae* was found to cause citrus dieback and gummosis (Ferrari et

al., 1996). In Japan, *Alternaria* spp. were identified as the causal agents of brown spot on citrus, which can also infect branches and cause dieback (Akimitsu et al., 2003). In Australia, *Fusarium* spp., *Phytophthora* spp., and *Phoma tracheiphila* were reported as the causal agents of citrus dieback (Tennant et al., 2009).

In a study conducted in California, *Fusarium* spp. (*F. solani*), the causative agent of citrus dry rot that leads to citrus dieback, was isolated (Adesemoye et al., 2011). Another study from India identified *Phomopsis citri* as a potential cause of citrus dieback (Mahadevakumar et al., 2014). In the California desert, *N. dimidiatum* and three species of *Eutypella* were found to be responsible for citrus dieback (Mayorquin et al., 2016). A study conducted across Greece, Italy, Malta, Portugal, and Spain revealed that *Diaporthe* spp. was associated with citrus dieback (Guarnaccia and Crous, 2017). In separate studies conducted in Greece, Italy, and Spain, certain *Fusarium* spp. and *Neocasmospora* spp. were identified as causative agents of dieback and canker in citrus branches (Sandoval-Denis et al., 2018). In Pakistan, a severe plant disease resulted in yield reductions of up to 40%, and *Colletotrichum siamense* and *Lasiodiplodia iraniensis* were identified as the causative agents of citrus tree dieback (Fayyaz et al., 2018). A new citrus disease displaying dieback-like symptoms was reported in California, with *Colletotrichum* spp. identified as the causal agent (Mayorquin et al., 2019). In Mexico, various fungal pathogens, including *L. theobromae*, *Fomitopsis meliae*, and *Eutypella citricola*, have been reported as causative agents of citrus dieback and other related symptoms (Polanco-Florián et al., 2019). An Egyptian study identified *Lasidiplodia* spp. as a causative agent of citrus mortality (El-Ganainy et al., 2022). These studies show that citrus dieback is a multifactorial disease that requires further investigation and management.

The first report of citrus dieback, with *N. mangiferae* as the causative agent, came from southern Iran (Keshavarz and Keveshk, 2016). In Kerman province, Iran, *N. mangiferae* has been identified as the causative agent of dieback in various trees, including orange, grapefruit, lemon, walnut, almond, pistachio, apple, peach, plum, cherry, eucalyptus, fig, and pomegranate trees (Aminaei and Ershad, 1993). *N. mangiferae* completely devastated over 100 hectares of Lisbon lemon trees within 3 to 5 years after symptoms appeared in the Dezful region of Khuzestan province (Alizade et al., 2000). In southern Kerman, two fungal species, *N. mangiferae* and *Bipolaris australiensis*, are known to cause branch drying in orange, grapefruit, tangerine, and lemon trees (Najafiniya, 2015). In various studies, different fungi such as *Cytospora* sp., *Fusarium* sp., *N. mangiferae* (Alizadeh et al., 2000), *Ceratocystis* sp., *L. theobromae*, *N. mangiferae*, and *Fusarium* sp. (Fateh et al., 2016) have been reported as responsible for citrus tree dieback and decline in Khuzestan. In Bushehr province, Iran, causative agents of citrus tree dieback were identified as *Spencermartinsia viticola*, *L. theobromae*, *N. hyalinum*, *Phaeoacremonium* spp. (including *P. parasiticum*, *P. rubrigenum*, *P. minimum* (*aleophilum*), and *P. alvesii*), *Cadophora luteo-olivacea*, *C. gloeosporioides*, *C. boninens*, *Pestalotiopsis* sp., and several species of *Phoma* (Espargham et al., 2020).

In light of the research conducted on citrus dieback in Kerman province, the primary objective of this recent study was to pinpoint the causative agents responsible for fungal dieback. The ultimate goal was to utilize these findings as a foundation for the development and implementation of effective management strategies.

MATERIALS AND METHODS

Sample collection

In the summer of 2020, sampling was conducted in citrus orchards located in Kerman province, Iran. Six citrus-producing districts within Kerman, namely Bam, Rigan, Narmashir, Fahraj, Shahdad, and Orzueeh, were surveyed (Table 1). Samples were systematically collected from 14 different orchards. Branches, bark, roots, stems, and leaves of citrus trees displaying symptoms such as dieback, canker, gumming, and decline were collected. These samples were carefully placed in plastic bags and transported to the laboratory.

Isolation of fungi associated with dieback

A 5 × 5 mm sample was excised using a scalpel at the junction between healthy and infected tissue within the specimen. Samples were obtained from both leaves and branches, subsequently sterilized using 70% ethanol for 5 seconds, 4% sodium hypochlorite for 90 seconds, washed with sterile distilled water for 60 seconds, and finally dried using filter paper. These samples were then cultured on both PDA (potato dextrose agar) and MEA (malt extract agar) media. Following isolation, fungi were purified through either the single-spore or hyphal tip method.

Pathogenicity test

Pathogenicity tests were conducted by artificially inoculating the purified fungi that had been previously

isolated on twigs of 2-year-old lemon seedlings grown in pots under controlled conditions. For each seedling, the thickest branch was selected for the inoculation of a fungal isolate. Subsequently, each isolate was inoculated onto three sections of the branch. To prepare the branch for inoculation, the surface was disinfected with 70% ethanol, the skin of the branch was carefully removed using a sterile blade, and a 6 mm mycelium-agar plug from a 1-week-old culture was placed into the wound. This area was then wrapped with parafilm to maintain moisture. Additionally, a cotton ball soaked in sterile distilled water was placed over the inoculated area and covered with parafilm. Non-colonized sterile agar plugs were used as controls. The inoculated plants were placed in a room with a temperature range of 21-26ºC and a lighting duration of 16 hours. After 6 weeks from the time of inoculation and the onset of symptoms, the branches were removed, examined for any changes in color, and the twigs were subsequently cultured (Guarnaccia and Crous, 2017).

Morphological Identification of fungi

Microscopic slides were prepared from proven pathogenic fungal strains, and the fungi were examined for characteristics such as spores, fruiting bodies, hyphae, spore shape, and color using an identification key (Dugan, 2006).

RESULTS

In this study, 46 fungal strains were obtained and purified from samples collected from 44 orange trees. Based on colony shape, culture characteristics and microscopic structure, the fungal strains were classified into 12 genera (Table 1). Pathogenicity tests revealed (Table 1) (Figure 1 and 2) that 10 genera caused disease symptoms on the seedlings, showing that they were virulent. The symptoms included leaf curling, yellowing, leaf drop, death, and twig dieback. The symptoms persisted for 6 weeks (Figure 3). Notably, the most significant symptom observed in infected trees was twig dieback.

Table 1. Fungi associated with dieback disease of citrus trees in Kerman province.

Pathogenicity test	Organ sampled	Fungal isolated	Site of sampling	Code of isolate
+	Twig	*Alternaria alternata*	Orzueeyeh	J_3
+	Trunk	*Nattrassia mangiferae*	Orzueeyeh	J_4
+	Twig	*Ulocladium* sp.	Orzueeyeh	J_5
+	Twig	*Fusarium* sp.	Orzueeyeh	$J_{5(1)}$
+	Twig	*Alternaria alternata*	Orzueeyeh	J_1a
+	Twig	*Alternaria alternata*	Orzueeyeh	J_1b
+	Twig	*Alternaria alternata*	Orzueeyeh	J_2b
+	Twig	*Phoma* sp.	Orzueeyeh	J_2b_1
+	Twig	*Nattrassia mangiferae*	Orzueeyeh	J_2d
+	Twig	*Chaetomium* sp.	Orzueeyeh	$J2d_1$
+	Twig	*Alternaria alternata*	Orzueeyeh	J_2a
+	Twig	*Fusarium* sp.	Orzueeyeh	J_2a_1
+	Twig	*Paecilomyces* sp.	Rigan	I_6
+	Twig	*Fusarium* sp.	Rigan	$I_{6(1)}$
+	Twig	*Alternaria alternata*	Rigan	$I_{6(2)}$
+	Twig	*Ulocladium* sp.	Rigan	$I_{6(3)}$
+	Twig	*Alternaria alternata*	Rigan	I_8
+	Twig	*Fusarium* sp.	Rigan	$I_{8(1)}$
+	Twig	*Ulocladium* sp.	Narmashir	H_5H_6
+	Twig	*Bipolaris* sp.	Narmashir	H_6
+	Twig	*Alternaria alternata*	Anduhjerd	A_5
-	Twig	*Chaetomium* sp.	Anduhjerd	$A_{5(1)}$
+	Twig	*Alternaria alternata*	Anduhjerd	Ba
+	Twig	*Stemphylium* sp.	Anduhjerd	A_2b

+	Twig	*Alternaria alternata*	shahdad	E_1
+	Twig	*Alternaria alternata*	Chaharfarsakh	C_3
+	Twig	*Acreonium* sp.	Chaharfarsakh	$C_{3(1)}$
+	Twig	*Alternaria alternata*	Anduhjerd	A_4
+	Crown	*Nattrassia mangiferae*	Fahraj	G_4
+	Twig	*Alternaria alternata*	Anduhjerd	Bb
+	Twig	*Bipolaris* sp.	Fahraj	G_6b
+	Twig	*Alternaria alternata*	Chaharfarsakh	C_2
+	Twig	*Nattrassia mangiferae*	Anduhjerd	A_2a
+	Twig	*Bipolaris* sp.	Narmashir	H_2
+	Twig	*Nattrassia mangiferae*	Anduhjerd	A_2d
+	Twig	*Alternaria alternata*	Rigan	I_2
+	Twig	*Alternaria alternata*	Narmashir	H_1b
+	Twig	*Ulocladium* sp.	Narmashir	H_1a
+	Twig	*Ulocladium* sp.	Fahraj	G_6a
+	Twig	*Rhizoctonia* sp.	Fahraj	$G_6a_{(1)}$
+	Twig	*Nattrassia mangiferae*	Fahraj	$G_6b_{(1)}$
+	Twig	*Bipolaris* sp.	Fahraj	$G_6b_{(2)}$
+	Twig	*Fusarium* sp.	Fahraj	$G_6b_{(3)}$
+	Twig	*Acremonium* sp.	Narmashir	$H_1b_{(1)}$
-	Twig	*Cladosporium* sp.	Narmashir	H_5
+	Twig	*Paecilomyces* sp.	Narmashir	$H_{5(1)}$

Out of the 46 strains obtained, three were found to be avirulent, including two strains of *Chaetomium* sp. and one isolate of *Cladosporium* sp. (Table 1). *Alternaria alternata* was the most frequently isolated fungal genus (Table 2) and was found throughout the citrus production area. In terms of pathogenicity, it ranked second after *N. mangiferae* according to the severity of symptoms. The symptoms caused on the seedlings were death and decline. However, it exhibited lower pathogenicity in terms of canker development at the infection site and discoloration of plant tissue compared to *N. mangiferae*. Symptoms caused by this fungus included dieback and severe defoliation (Figure 3). Additionally, when the skin was removed from the infected area, the wounds exhibited a range of colors from dark brown to black. These lesions were concave and larger than those caused by other fungi (Figures 4, J4 and G4) (Table 2).

Table 2. Percentage of each fungal genus isolated from orange trees.

Sr. No.	Identified fungi	No. of isolates	Percentage	Dimension of canker in cm
1	*Alternaria* spp.	16	34.78	0.53×1.56
2	*Nattrassia mangiferae*	6	13.04	1.53×1.53
3	*Ulocladium* spp.	5	10.86	0.46×1.53
4	*Fusarium* spp.	5	10.86	0.5×1.53
5	*Bipolaris*spp	4	8.69	0.53×1.46
6	*Paecillomyces* spp.	2	4.34	0.5×1.53
7	*Acremonium* spp.	2	4.34	0.5×1.5
8	*Chaetomium* sp.	2	4.34	-
9	*Phoma* sp.	1	2.17	0.43×1.16
10	*Stemphylium* spp.	1	2.17	0.5×1.6
11	*Rhizoctonia* sp.	1	2.17	0.5×1.5
12	*Cladosporium* spp.	1	2.17	-

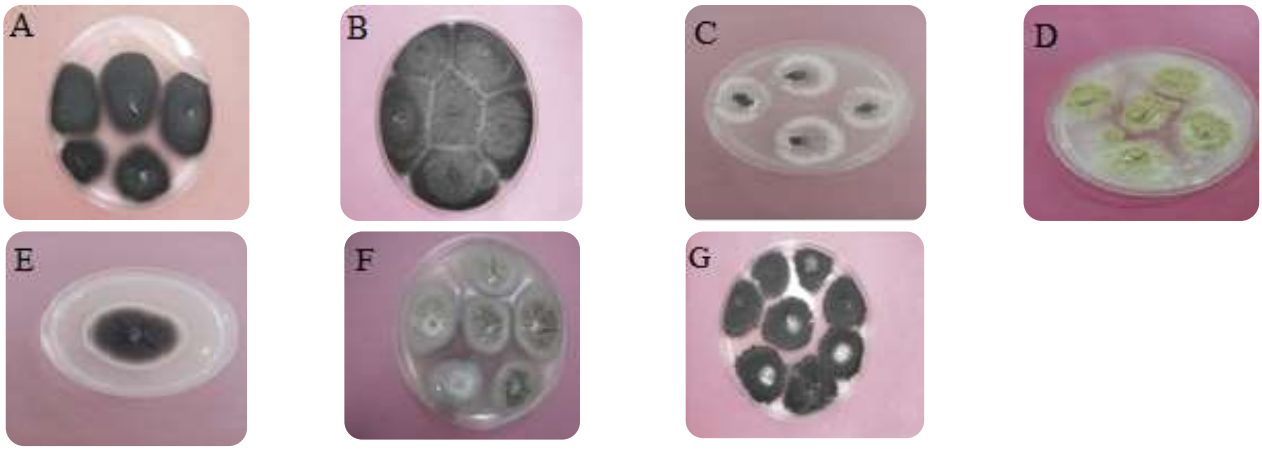

Figure 1. Fungal colonies isolated from citrus trees. A: *Alternaria* spp.; B: *Bipolaris* sp.; C: *Nattrassia mangiferae*; D: *Paecilomyces* sp.; E: *Phoma* sp.; F: *Stemphylium* sp.; G: *Ulocladium* sp.

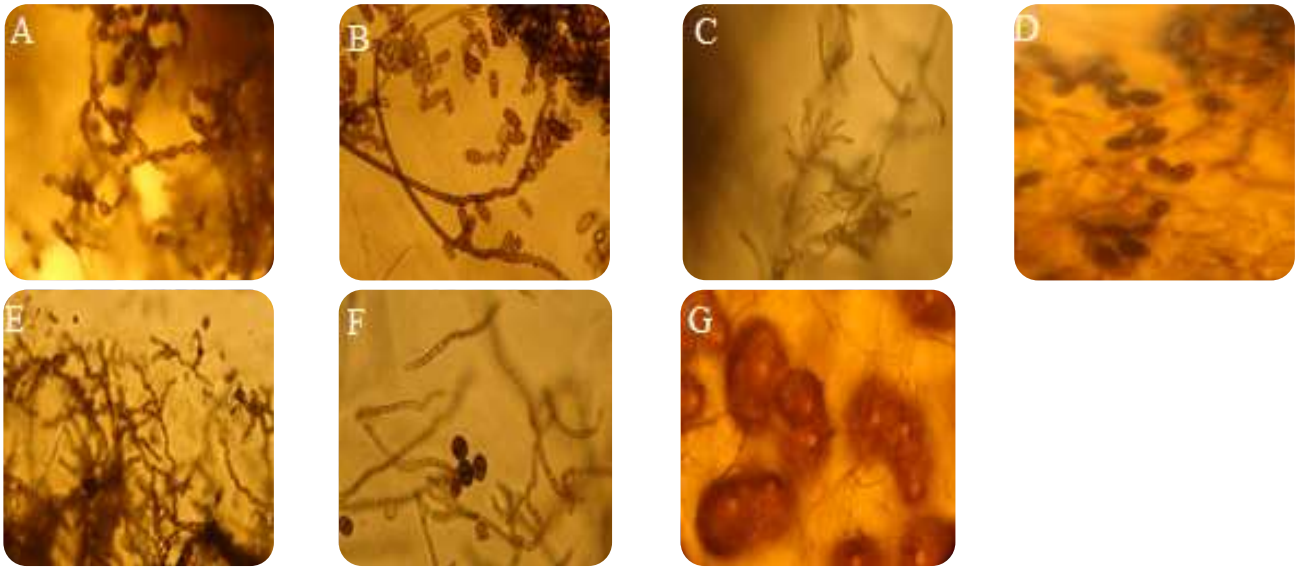

Figure 2. Conidia of isolated fungi. A: *Alternaria* spp.; B: *Bipolaris* spp.; C: *Paecilomyces* spp.; D: *Stemphylium* spp.; E: *Nattrassia mangiferae*; F: *Ulocladium* spp.; G: Pycnia of *Phoma* spp.

Figure 3. Symptoms on seedlings after fungal inoculation. A: Canker; B: Defoliation; C: Dieback

Bipolaris sp. and *Ulocladium* sp. also caused dieback and severe defoliation. These fungi followed the two already mentioned in terms of frequency and pathogenicity. Other fungal genera such as *Acremonium* sp., *Paecilomyces* sp., *Stemphylium* sp. also caused dieback symptoms, but their severity was less. In general, these fungi were less important than the other genera mentioned in terms of disease frequency and intensity due to the symptoms they caused in plants. In this test, the control was asymptomatic and no fungus was ultimately isolated from it. In addition, the transplant resulted in no abnormal color change and the wound was due to the plant's response to cutting (Figure 4: A).

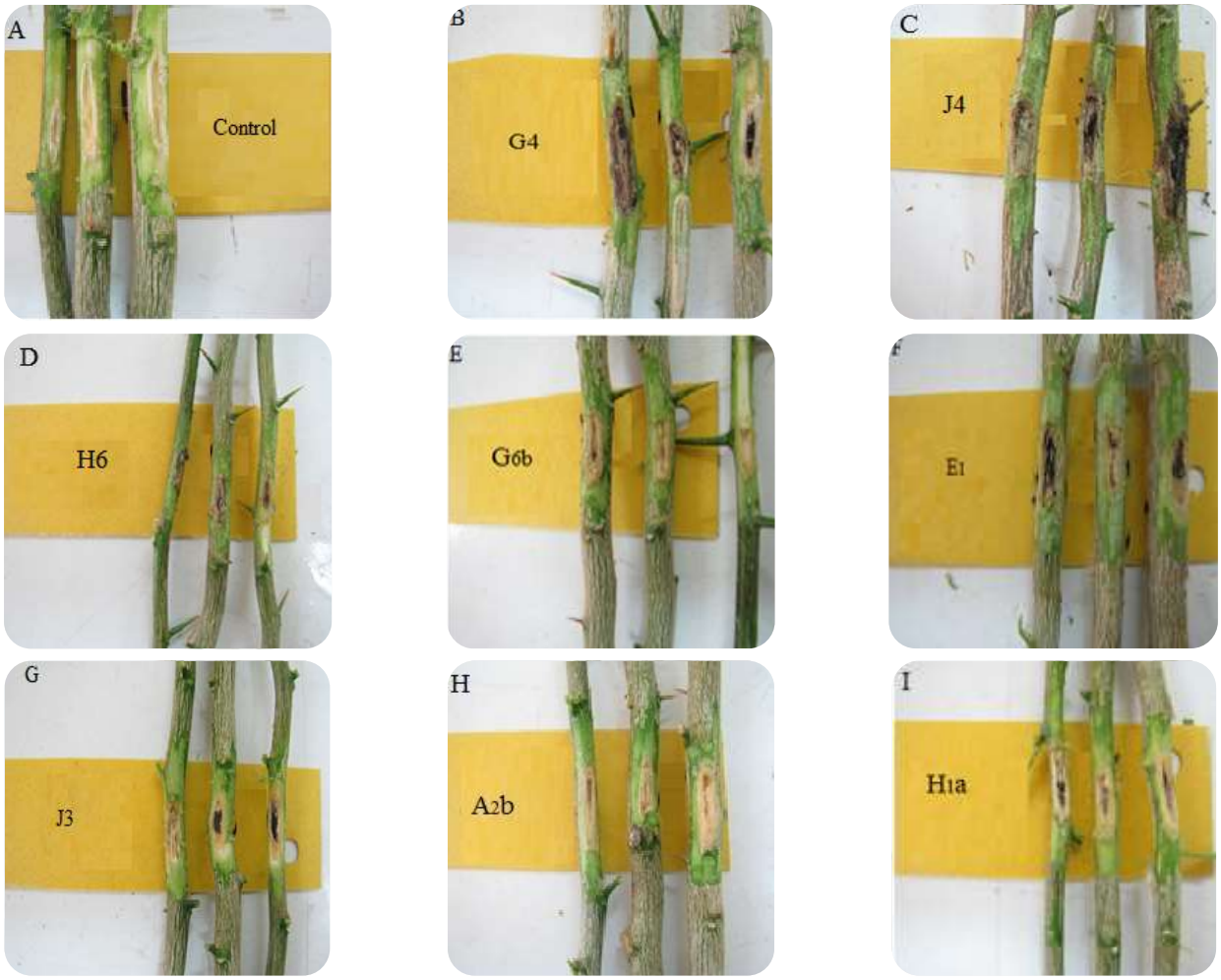

Figure 4. Canker and discoloration caused at the inoculation site after 6 weeks. A: Control; B and C: *Nattrassia mangiferae*; D and E: *Bipolaris spp*.; F and G: *Alternaria* spp.; H: *Stemphylium spp.*; I: *Ulocladium* spp.

DISSCUSSION

According to the results of this survey, all citrus orchards in Kerman province are facing the problem of dieback, wilting and decline. These complications have led to reduced citrus yields. Citrus decline has become a complex problem on a global scale. This complication is a combination of many different factors. Therefore, it cannot be attributed to a specific factor. According to the results of various studies conducted in different countries of the world, this complication is the result of a number of living and non-living factors (Irshad et al., 2012; Fateh et al., 2017; Saeed et al., 2019). Non-living factors include soil texture, structure, nutritional issues, moisture levels, and physiological disorders, while living factors involve parasitic plants, insects, nematodes, and plant pathogens such as fungi, bacteria, and viruses (Meena et al., 2018).

To manage citrus dieback, it is crucial to detect and

identify the causative agent. In this study, only fungal pathogens were monitored. Furthermore, the distribution and spread of the fungal agent across all regions of Kerman province were nearly uniform, with samples collected from citrus fruits in different areas. The most prevalent fungus in terms of frequency and widespread distribution was *Alternaria* spp., which has also been reported by other researchers as a cause of dieback in kiwifruit and other plants (Tsahouridou and Thanassoulopoulos, 2000).

In a recent study, the most significant fungus identified was *N. mangiferae*. This fungus has a broad host range and can induce disease under favorable weather conditions, characterized by high humidity and temperature. *N. mangiferae* is believed to be responsible for canker and wilt in various tree species, including Apricots, Almonds, Apples, Grapes, Pistachios, Eucalyptus, Berries, Pears, Citrus fruits, Walnuts, and Figs across different regions of the world. This fungus operates as a wound parasite, with sunburn being a contributing factor that makes trees more susceptible to it (Mirzaei et al., 2002). Recent research has revealed that *N. mangiferae* is the primary cause of declining and dying citrus tree branches in tropical provinces of Iran, including Khuzestan, Fars, and Kerman. Studies have also demonstrated that older trees, especially those that have experienced mechanical damage or sunburn, are more vulnerable to this fungus than young, healthy trees (Keshavarz and Keeves, 2016). Apart from *N. mangiferae*, other fungi such as *Fusarium* spp., *Acremonium* sp., and more can contribute to plant decline (Fateh et al., 2016).

The present study involved the isolation of various species of *Fusarium* from tree branches, among other fungi, during the initial isolation process. Upon inoculation in citrus seedlings, their pathogenicity was confirmed. The isolation of *Fusarium* spp. from citrus branches may indicate the presence of root infection (root rot) in these trees (Adesmoy et al., 2011). In Punjab, Pakistan, *Fusarium oxysporum* and *F. solani* were documented as the most prevalent species, along with other contributing factors responsible for the decline of citrus trees (Ali et al., 2014).

The fungi isolated in this study, such as *Ulocladium* sp., *Paecilomyces* sp., *Stemphylium* sp., *Alternaria* spp., *Fusarium* spp., and *N. mangiferae*, were all obtained from pistachio trees displaying symptoms of twig dieback (Ghelichi et al., 2012). *Cladosporium* sp. and *Chaetomium* sp. were also isolated in this study; however, these fungi are non-pathogenic and can be considered citrus endophytes. In other studies, these genera have been reported as endophytes in citrus (Nicoletti, 2019). The general result obtained from this study also indicates an association of several factors contributing to the death and decline of citrus fruits. Both living and non-living factors, such as nutritional deficiencies, toxicity, other pathogens (fungi, bacteria, viruses, and citrus nematodes), improper irrigation, poor soil texture and structure, and pests, are important contributors. The pathogenic fungi identified in this study are not strongly parasitic; they are mainly considered secondary pathogens and highly saprophytic. The weakness of the host plant due to various reasons, such as water and nutrient deficiency, mechanical damage, etc., can act as a co-factor in citrus tree dieback and decline due to the fungal agents mentioned above.

Depending on the type of fungus identified in this study, several citrus dieback management methods may be recommended, such as proper irrigation and fertilization. Additionally, pruning dead branches, disinfecting the pruning site with copper oxychloride or Bordeaux mixture, and removing pruned tree residue from the garden can help reduce this disease. In the case of pistachio dieback disease (Ghelichi et al., 2012) caused by *N. mangiferae*, *Stemphylium* spp., and *Paecilomyces variotti*, three fungicides, namely Rovral T-S, Propiconazole, and Bordeaux mixture, were used and found to be effective. Therefore, these fungicides could be considered for testing against citrus dieback after this study. Based on the results of this test, the mentioned fungicides may be recommended as a potential solution for managing citrus dieback.

AUTHOR'S CONTRIBUTIONS

MR designed the research, conducted surveys, isolated and identified fungi, carried out all the experiments, wrote and proofread the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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