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# First Report of *Fusarium oxysporum* Causing Fusarium Wilt on *Thuja plicata* in Iran

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Abstract

*Thuja* is one of the ornamental plants belonging to the conifers and Cupressales order. Coniferous trees are always important as creators of urban green spaces in terms of environmental values due to practical reasons and fast growth. In recent years, thuja wilt disease has caused irreparable damage to urban greenery, and its symptoms appear in the form of weakness and drying of the branches, which eventually causes the death of the tree. This research was conducted in order to investigate the causes of root and collar drying of thuja (*Thuja plicata*) in the west of Guilan province, during 2021-2022. In this study, samples were taken from the western regions of Guilan province. 40 samples were taken from the infected parts of the root and collar. After culturing the infected parts on the culture media, five fungal isolates were isolated in this study, the pathogenicity of this fungus was confirmed on potted thuja seedlings. Out of five isolates, two isolates showed wilting and rotting of the root and drying of the tips of the leaves with the same intensity of pathogenicity. These two isolates were similar in terms of their shape, and only one isolate was subjected to further laboratory work. Different cultures and molecular methods were used to identify this fungus. Based on the identification key and the molecular method, the disease-causing fungus *F. oxysporum* was identified.

**Keywords:** Conifer, Fusarium, Guilan, Thuja.

## INTRODUCTION

Thuja is a genus of coniferous trees or shrubs from the Cupressaceae family (Farjon, 2005). Decay disease of cedar trees has existed in different regions of the world for nearly a hundred years, and various factors have been reported for it. In 1943, investigations were conducted on the drying of cedar leaves, and *Sphaeropsis sapinea* isolates were reported as the cause of leaf drying (Waterman, 1943). Thuja is susceptible to fungal diseases such as root rot and collar rot, Fusarium and Septoriose, which affect the branches and needles of the plant. *Fabrella thujina* fungus causes the needles to turn brown, *Coryneum beckmanii* causes the tips of the needles to dry out (Herfeh Dost, 2008). Root and collar rot disease of thuja is one of the most important diseases of thuja that causes significant damage to this product, since Fusarium species play a role in causing the disease, therefore, identifying these species on thuja is important (Gleason, 1963). A number of fungal and pseudo-fungal pathogens including *Fusarium circinatum*, *Phytophthora parasitica*, *Ph. cinnamomi*, *Ph. boehmeriae* and *Pythium splendens* have been reported as greenhouse pathogens of needles leaf (Linde *et al*., 1994; Dick and Dobbie, 2002).

Various species of *Pythium*, including *P. vexans* and *P. oligandrum*, have been reported as the causes of needle mortality in needles leaf (Ershad, 2009). Investigations show that in more humid areas, some basidiomycete fungi may also attack the needles, such as *Fomes annosus*, *Collybia velutipes*, and *Tremetoes betulinia* fungi have been reported (Ershad, 2009). During the years 1988-1991, the fungi *Macrophomina phaseolina*, *Phytophthora cactorum*, *Ph. citrophthora*, *Ph. citricola*, *Fusarium solani*, *F. oxysporum*, *F. moniliforme*, *F. avenaceum*, *Rhizoctonia solani*, *Pythium* spp., *Ph. nicotianae* var. *parasitica*, *Ph. drechsleri*, *Ph. cryptogea*, *Cylindrocarpon radicicola* isolation and pathogenicity of the species have been proven (Mirabolfathy and Ershad, 1996).

The rotting of seeds and roots of pine and cypress has caused heavy losses to nurseries in Fars province, and in a survey conducted in 2011, Pythium, Rhizoctonia, Phytophthora and Fusarium fungi were isolated from the collar and root of various trees, such as species of pine and cypress, which had symptoms such as chlorosis, necrosis, growth arrest and collar and root rot. In this research, Fusarium fungus was isolated from pine and cypress seeds. A species of *F. proliferatum* caused decay on cypress seeds before germination (Zakeri *et al*., 2011). In Guilan province, no research has been done in the field of identifying the fungal agents involved in the drying and rotting of the roots and collar of cypress trees, including thuja, and considering the importance of this tree in Guilan province, identifying the fungal agents involved in the drying and rotting of the roots and collar of cypress trees is important and necessary.

## MATERIALS AND METHODS

### Sampling and assessment

A fixed plot survey was taken up during 2021-22, to know the incidence of diseases, *Thuja plicata* in West of Guilan province in Iran districts. The disease incidence was assessed by recording the number of plants showing disease symptoms and the total number of plants present. In each nursery, rows were selected randomly and the number of plants showing typical symptoms and the total number of plants were recorded (Wheeler, 1969).

### Medium

Purification and identification of the pathogen was cultured, PDA and CLA. Key on

the mycological evaluated growth and generative structures, the fungal isolates were identified (Nelson *et al*., 1983).

**Isolation of pathogens**

The infected plant parts were surface sterilized with 1:1000 mercuric chloride ($HgCl_2$) solution for 30 seconds and washed separately in sterilized distilled water twice to remove the traces of mercury, if any and then transferred to sterilized Petri plates containing potato dextrose agar (PDA). The Petri plates were incubated at room temperature 25 ºC and observed periodically for the growth of pure colonies. The pure colonies which developed from the bits were transferred to PDA slants and incubated at 25 ºC for 15 days. Then such slants were used to study the cultural characters in the laboratory (Ben-Yephet *et al*., 1994).

**Identification**

This method was followed for maintaining pure cultures. Dilute spore suspensions (8-10 macroconidia /ml) of the isolated pathogens (*F. oxysporum*) were prepared in sterile distilled water. One ml of such suspension was spread uniformly on two per cent water agar plates and the excess of which was aseptically drained. Such plates were incubated at 25 ºC and periodically observed for germination of spores under the microscope. Hyphae coming from each end cell of the single spore was traced and marked with the ink on the reverse side of petri plates. Then tip of hypha was cut and transferred to PDA slants with the help of cork borer under aseptic conditions and incubated at temperature of 25 ºC for 10 days. Later, mycelial bits of the fungus were placed in the center of Petri plates containing potato dextrose agar medium and incubated at 25 ºC for 10 days. No saltation or sectoring was observed in the culture and it was concluded that, it was a pure culture of the fungus. The spores of the pathogens were taken from infected plant portions (collar region) and temporary slide mounts were prepared in lactophenol. Then they were observed under high power objective (40x). One hundred spores (macroconidia, microconidia and chlamydospores) of the pathogens were observed under microscope and measured using ocular and stage micrometer (Nelson *et al*., 1983).

In molecular studies, PDA medium was used to form mycelial masses. DNA extraction it was done with CTAB method (Gardes *et al*., 1991). The ITS4-ITS5 regions of nuclear ribosomal DNA were amplified using primer set (ITS4 (TCCTCCGCTTATTGATATGC) and ITS5 (GGAAGTAAAAGTCGTAACAAG)) combinations by polymerase chain reaction (Gardes *et al.*, 1991). In order to compare and check the similarities and differences of the protected sequences of the fungal isolates with the sequences recorded in the gene bank (GenBank; NCBI), the PCR product of the ITS region of the different isolates was sent to Takaposist company for sequencing. The sequences obtained using Mega software were lined up at the end after blasting and comparing the results of comparing the nucleotide synonymy of the fragment amplified from the ITS region and using gene bank data, and using the maximum likelihood method, its phylogenetic tree was drawn using MEGA11 software (Sedaghatfar *et al*., 2012; Mohammadrezabeigi *et al*., 2021).

**Proving pathogenicity by inoculating**

Dipping the roots in a spore suspension Roots was placed in a spore suspension to concentration of $10^6$ for 25 minutes. The seedlings were grown in pots with a diameter of 14

cm. The roots of control seedlings were placed in water. Then seedlings in pots with a diameter of 14 cm were cultured in pasteurized soil. The pots were kept for 1 month in the greenhouse conditions. Discolored, yellow and faded plants (leaf chlorosis symptoms) (percent wilting) were recorded (Carver *et al*., 1996).

## RESULTS

### Sampling and assessment

This research was carried out in 2021-2022 in the western regions of Guilan province (Foman and Someh Sara) of Iran. 40 samples of infected plants were taken from nurseries and sent to the laboratory for identification. The diagnosis of this species of Fusarium was done using reliable sources and the identification key of Nelson *et al.* (1983). In the case of single-spored isolates, it was done based on the following characteristics.

### Isolation of pathogens

This fungus was isolated from thuja root and collar using Nash and Snyder (1962) culture medium. The color of the colony from the bottom surface on the PDA culture medium was variable from white, purple to dark purple. The aerial mycelium on the culture medium were relatively abundant and cotton-like at first, and then changed from pink to pale purple, although in some isolates, aerial mycelium were formed in a very small amount(Fig. 1).

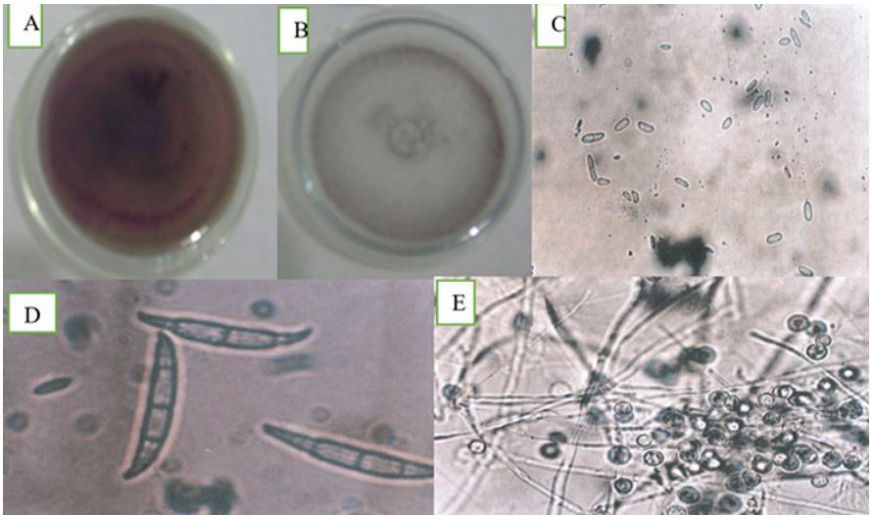


Fig. 1. Colony image of *F. oxysporum*. A: Upper surface, B: The back surface, C: Microconidia one-celled or two-celled. (x100), D: Sickle-shaped macroconidia with a hook-like terminal cell (60x), E: Chlamydiospores are round with a thick wall.

It sporulation easily on PDA and CLA cultures after 2-3 days. At first, only micro conidia were produced from the short lateral phialides located on the aerial mycelium, which were false heads, but later macro conidia were also produced. Microconidia were abundantly formed and were unicellular and bicellular. They were found in oval, egg-shaped to almost club-shaped shapes, but they were mostly oval. The two ends of the macro conidia were slightly narrowed, their terminal cell was slightly pointed and their basal cell was relatively stalked. Most macroconidia are three-walled, but sometimes there are four-walled ones (Fig. 1). The size of the conidia was as follows:

Unicellular microconidia: (2-3) 2.2 x (4-10.5) 6.5 micrometers - Two-cell microconidia: (2-3.2) 2.4 x (11.2-4.3) 7.2 micrometers - Three-walled macroconidia: (3.4-4.2) 3.7 x (20-40) 35 micrometers - Four-walled macroconidia: (3.4-4.3) 3.8 x (30-42) 38 micrometers - Five-walled macro conidia: - (3.4-4.3) 3.8 x (36-48) 41 micrometers.

Chlamydospores hyphae and conidiophores are abundantly formed in middle and end form. In most isolates, they have a smooth surface, but in some, they have a rough and uneven surface. They were formed in a spherical shape, singly, in pairs, short chains and sometimes in a mass. Based on the key of Nelson *et al.* (1983), the mentioned characteristics corresponded to *Fusarium oxysporum* species (Fig. 1).

**Proving pathogenicity by inoculating**

In this study, among the 5 fungal isolates isolated from thuja root, 3 treated pots, some degree of wilting and rotting of the root and drying of the leaf tips were seen, and 2 isolates were similar in terms of the shape of the leaf, which continued the laboratory work on only one isolate and was named 3F-. In none of the control pots, signs of yellowing of the leaf tips, root rotting were not seen. In the microscopic studies, the fungus isolated from the roots with disease symptoms was the same as the crushed yeast mushrooms. In order to comply with the principles of Koch, infected roots were cultured on PDA culture medium after disinfection. After isolation and purification, the fungus causing the disease was re-diagnosed.

**Molecular studies**

After extracting DNA genomic, internal transcribed sequence region was amplified using ITS5 and ITS4 primers. Using blast search tool, the isolate of *Fusarium oxysporum* in the gene bank had 99 % overlap and high similarity. Three most similar sequences from the ITS region of isolates of *F. oxysporum*; small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence, were selected for phylogenetic analysis included; isolate A549-genbank: KX463005.1, isolate QX-1 ON416887.1 and isolate XJ-6: ON394593.1. The phylogenetic results confirmed the correct molecular and morphological identification of the species (Fig 2).


Fig 2. Molecular phylogenetic analysis of *F. oxysporum* from Thuja by maximum likelihood method comparing with 3 accession from genbank.

Ancestral states were inferred using the maximum likelihood method (Nei and Kumar, 2000) and Tamura-Nei model (Tamura and Nei, 1993). The tree shows a set of possible nucleotides (states) at each ancestral node based on their inferred likelihood at site 1. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. The rates among sites were treated as being uniform among sites (Uniform rates option). This analysis involved 4 nucleotide sequences. There were a total of 570 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura *et al*., 2021).

## DISCUSSION

*Thuja plicata* (Cupressaceae) is an important medicinal tree found in tropical and subtropical countries. Evergreen tree is rich in borneol, borneol acetate, thujone, camphor and sesquiterpenes (Dan and Nhu, 1989). The leaves are antibacterial, antipyretic, antitussive, astringent, diuretic and have hemostatic and cooling effects and are used to treat bronchitis, asthma, skin infections, mumps, bacterial dysentery and arthritic pain (Brown, 1995).

One of the major problems that causes a lot of damage to nurseries in humid areas of the country every year is the disease of seedlings. One of the causes of this disease is the presence of soil-borne fungi that live a saprophytic life and gradually damage the lower organs of the plant and lead to their withering, so that the affected seedlings show signs of wilting, root rot and yellowing of the needles six months to one year after planting. Unfortunately, due to the weak management of the nurseries, other factors aggravate this disease. Failure to observe points such as the correct principles of irrigation, lack of proper drainage in the planting beds, salinity of the nursery soil and the resulting poisoning have been imposing many costs on our nurseries for years (Zakeri *et al*., 2011; Oono *et al*., 2015).

This study was conducted in Foman and Someh Sara nurseries located in the West of Guilan province, in order to determine the disease agent of thuja seedlings in the region. By carrying out the pathogenicity test, based on the morphological and molecular data from ITS region of the isolate of this research was identified as *Fusarium oxysporum*. In India, in 2007, this fungal agent was reported as the cause of wilting of thuja tree (Raghavendra, 2007). Currently, many studies have been conducted on the diseases of cedar trees in different parts of the world, and various fungal agents have been isolated as the cause of the disease. The results of this survey showed that the dieback disease of cypress trees is present in most parts of Foman and Someh Sara and is progressing. The main fungi that cause the death of seedlings are: Fusarium, Rhizoctonia, Pythium and Phytophthora (Cram, 2003). As it can be seen, the fungi obtained in this study are among the main fungi that cause the death of seedlings. *Fusarium solani* has the highest abundance percentage among the Fusarium fungi obtained in the studies conducted by Herfe Dost in Lakan region (Herfe Dost *et al.*, 2008). The mentioned species is one of the pathogenic fungi that have been introduced from different parts of the world as one of the causes of seedling death, and it is usually seen with other fungi such as Pythium, Rhizoctonia and Phytophthora or with other species of the Fusarium genus, *F. oxysporum* as well as *F. solani* has a global distribution (Nelson *et al.*, 1983).The morphological and physiological characteristics of this species are associated with severe changes in the cultivation environment (Burgess, 1981). This is probably the reason that this species is able to occupy wide ecological areas in many geographical areas. This species has specific forms and different populations and

is able to cause disease in many plants. Herfe Dost *et al*. (2008) was reported *F. oxysporum* for the first time in Iran as the cause of death of seedlings of needle leaf.

## CONCLUSION

This species is reported for the first time in Foman and Someh Sara region as the cause of death of thuja seedlings. Due to the lack of proper irrigation, the hardness of the soil at the base of the trees, and the lack of aeration of the soil, conditions are provided for the pathogenicity of opportunistic fungi and, in some cases, endophyte fungi becoming pathogenic.

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