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https://doi.org/10.5109/7236819

出版情報：Evergreen. 11 (3), pp.1655-1665, 2024-09. 九州大学グリーンテクノロジー研究教育センター
バージョン：

Morphological and Molecular Identification of *Fusarium Oxysporum* as the Main Cause of Wilt Disease of Tomatoes Grown in Greenhouses in Albania

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(Received April 26, 2024: Revised July 24, 2024: Accepted August 2, 2024).

Abstract: When growing tomatoes indoors, Albanian farmers face plant diseases caused by the endophytic fungus *Fusarium oxysporum*. This causes significant losses to farms and reduces their export potential. The study aims to review the methods for identifying this pathogen and assess its prevalence, as well as the feasibility and conditions for using nucleotide sequence analysis in its identification. Damaged root and shoot tissues from 50 plants with signs of the disease were collected in the regions where tomatoes are most widely grown. The samples were surface sterilised and placed on potato dextrose agar (PDA). Incubation was carried out at 25°C for 7-10 days. Next, individual target colonies were isolated, and their cultural and morphological identification was performed, supported by light microscopy using an immersion objective. The colour, shape and texture of the colonies, their growth dynamics, time of emergence and sporulation intensity were considered. For the strains selected in this way, the nucleotide sequence was determined by polymerase chain reaction and compared with those in the GenBank database of the National Centre for Biotechnology Information using BLASTn search. As a result, it was determined that all affected plants contained strains of *Fusarium oxysporum*. At the same time, the use of nucleotide sequence analysis revealed inaccuracies in the preliminary identification by traditional methods. Thus, the expediency of additional measures for strain identification was determined, when conducting research for which high species specificity is critical. It was also found that farmers in Albania need to take measures to identify the strains of this fungus in plant tissues and soil on which they are grown.

Keywords: phytopathogens; *fusarium*; indoor conditions; strain isolation; identification of microscopic fungi; endophytic fungi

1. Introduction

Tomatoes (*Solanum lycopersicum L.*) are a widespread crop grown in many countries around the world, including Albania, which is an economically important crop. Tomatoes, together with cucumbers, are the main vegetables for export in this country[1)]. Although tomato is considered an unpretentious plant that is not prone to frequent diseases, in places where these vegetables are grown year after year, and especially in greenhouses, the likelihood of crop damage by various pests, phytopathogens and viruses increases significantly[2)]. Fungal diseases account for a significant proportion of these. Phytopathogens such as *Phytophthora nicotianae, Pythium spp, Botrytis cinerea, Leveillula taurica, Fusarium oxysporum, Didimella lycopersici, Cladosporium fulvum* are particularly common[3)]. *Fusarium oxysporum* is one of the most widespread soilborne deuteromycetes, with about 120 species, some of which are phytopathogens. Many of them are host-specific.

One of the diseases is tomato wilt or *Fusarium* wilt, caused by *Fusarium oxysporum* f. sp. *lycopersici*. It results in large economic losses all over the world[4)]. The plant becomes infected with the illness, which blocks water and nutrients and causes wilting, stunted growth, and eventually death. Diminished yields, escalated production expenses, and disturbances in the market are examples of direct economic losses. Infestations that persist over time can harm soil quality and threaten financial stability[5)]. J. Li et al. note that this strain was first described in England in 1895, and since then it has been found in more than 40 countries[6)]. N. Mukjang et al., described the life cycle, which begins with root invasion, then gradual colonisation of the xylem, vascular occlusion and release of specific toxic substances, which leads to severe stress, up to the death of the host[7)]. K. Srinivas et

al. identified such external syndromes as blackening of vascular fibre bundles, wilting, spotting and curling of leaves, starting from the lower ones, dark spots on the roots or their complete darkening[8].

As noted by S. Panno et al., *Fusarium oxysporum* f. sp. *lycopersici*, which is widespread in the Mediterranean basin countries, including Albania, can cause yield losses of up to 70%[9),10]. The taxonomy of *Fusarium oxysporum* is based on several morphological features, namely, as noted by M. Torbati et al., it considers the shape and size of macroconidia, the type of anamorph, the presence or absence of chlamydospores and microconidia, the characteristics of the conidium, and the colour of the colony[11]. The first stage of treatment is usually the identification of the phytopathogen to apply the most effective drugs. The easiest way is to identify it by external symptoms, but quite often pathogens have a similar effect on the affected plant, and therefore the external signs will be similar. In addition, as the authors note, the diversity of fungi in the genus is not yet deeply understood, nor is the interaction of *Fusarium* with other microorganisms. For identification, however, the standard approach is to isolate the phytopathogen on a suitable nutrient medium into a pure culture and perform a microscopic examination[12].

K. Vignesh et al. determined the strains of *Fusarium oxysporum* f. sp. *lycopersici* isolated from tomatoes by selecting isolates from potentially affected plant tissues with signs of disease, further morphological characterisation, and analysis of genetic material[13]. They note that although the high host specificity of this fungus facilitates identification, it is hampered by the morphological similarity or even identity of saprophytic and phytopathogenic strains.

The study aims to identify the cause of the disease in tomatoes grown in closed ground, to isolate and culture, microscopically and morphologically identify the pathogen, and to compare the nucleotide sequence fragments of the isolates with the GenBank data of the National Center for Biotechnology Information (NCBI) to confirm the species identity.

2. Materials and methods

Plant samples for pathogen isolation were collected in the protected areas of Berat and Lushnja, the areas with the most intensive tomato cultivation. Among the plants grown in these areas, 50 were selected that visually showed symptoms of the disease. Stem and root tissue samples were obtained from these plants. To isolate *Fusarium* sp., root and stem tissues were washed under running tap water. Next, the washed tissues were surface sterilised in a 1% NaOCl (sodium hypochlorite) solution for 1-2 minutes, then rinsed twice in sterile distilled water and placed on similarly sterile filter paper, covered with a similar sheet on top to dry. For further work, the surface-disinfected tissues of the lower hypocotyls and taproot were cut and placed in pre-prepared Petri dishes with potato dextrose agar (PDA), to which streptomycin sulfate (200 mg/l) was added at the preparation stage. The plates with samples were incubated at 25°C for 7-10 days, sufficient for the development of fungi and inhibition of the growth of endophytic bacteria, which develop faster on nutrient media[14].

The fungal hyphae that appeared on the selected plant tissues were transferred to a newly prepared PDA medium using the hyphal tip transfer method. The most representative fungal colonies were purified by three subsequent inoculations on PDA, selecting cells from the edges of the colonies. The isolates were examined for cultural characteristics and conidial morphology. Colonies with taxonomic features of *Fusarium* sp. were identified according to P.E. Nelson et al.[15]. To confirm the correctness of the identification, morphologically representative fungal isolates, which were observed most often during the first stages of incubation, were sent for further genome sequencing. For this purpose, it was decided to extract the internal transcribed spacer (ITS) and the gene for translation elongation factor 1-alpha (TEF1). Subsequently, the complete genomic DNA was isolated using a specific technique[16]. The ITS regions 1 and 2, which also included 5.8S ribosomal RNA, were amplified by polymerase chain reaction (PCR) using primers ITS1 and ITS4, while a fragment of the TEF1 gene was amplified using primers EF1-1018F and EF1-1567R[17)-19].

For the reaction, a 512-model thermocycler manufactured by Techne, containing a T-gradient, was used. The parameters of the thermal cycling programme were set in advance: 2 min at 94°C corresponded to the denaturation process, for the next 30 s the temperature was reduced by 2°C and for the same period to 55°C, then the temperature was finally set at 75°C, first for 60 s for 30 cycles, and then for another 5 min for the final extension. The amplicons were sequenced with the help of Eurofins Genomics, a German company based in Ebsberg. Identification of species for each polymerase chain reaction product was performed by searching the sequence data in the GenBank of the National Center for Biotechnology Information (NCBI) using the BLASTn search engine. The results of morphological and cultural preliminary identification were compared with the results of nucleotide sequence comparison.

The goal of the study was to apply statistical techniques to detect *Fusarium oxysporum* f. sp. *lycopersici* in tomatoes. The frequency and distribution of *Fusarium* sp. colonies from symptomatic plant tissues were compiled using descriptive statistics, which gave a clear picture of the data and pointed out any noteworthy trends or abnormalities. Fungal isolates were categorised and identified using morphological analysis according to their physical attributes, including size, shape, colour, and growth patterns. This offered an initial categorization that molecular methods might validate. The most important statistical technique was DNA sequencing, which involved utilising the BLASTn programme to compare the amplified fungal DNA sections to known sequences in

the National Centre for Biotechnology Information (NCBI) GenBank. This method outperformed morphological approaches in terms of resolution, offering a high degree of accuracy and specificity in the identification of *Fusarium oxysporum*. Combining these techniques produced a thorough and accurate diagnosis of *Fusarium oxysporum* f. sp. *lycopersici*, which laid the groundwork for deciphering the pathogen's effects on tomato crops and developing practical management approaches.

According to the study, the primary pathogen responsible for the wilt symptoms in tomatoes planted in protected regions of Berat and Lushnja was *Fusarium oxysporum* f. sp. *lycopersici*. Fusarium sp. colonies were consistently seen in isolates from root and stem tissues out of 50 samples; these colonies were further categorised into five primary morphological kinds. The species identification was verified by DNA sequencing, since four morphotypes matched sequences of *Fusarium oxysporum* found in the NCBI GenBank database. This thorough method produced precise species identification and verified preliminary classifications.

3. Results

Depending on the area and intensity of tissue damage, the number of colonies obtained on PDA petri dishes varied: with an increase in the manifestation of the disease, the species diversity of microorganisms decreased, and the proportion of morphologically representative colonies of *Fusarium* sp., on the contrary, increased. When compared visually and quantitatively, root endophytes were more diverse than stem endophytes, and target colonies of *Fusarium* sp. were more frequent in them. The same relationship was observed between the base of the stem and the areas above: with distance from the root, the diversity of all, and target colonies decreased, as did their number. There was also a tendency that the morphological variation of *Fusarium* colonies was less dependent on the location of the tissues from which isolation was performed, but more often differed from plant to plant and from the specific farm from which the samples were taken. After completion of the final stages of visual identification and mechanical sieving and isolation, 5 main morphological types of colonies were identified as probably belonging to *Fusarium oxysporum* (Figure 1).

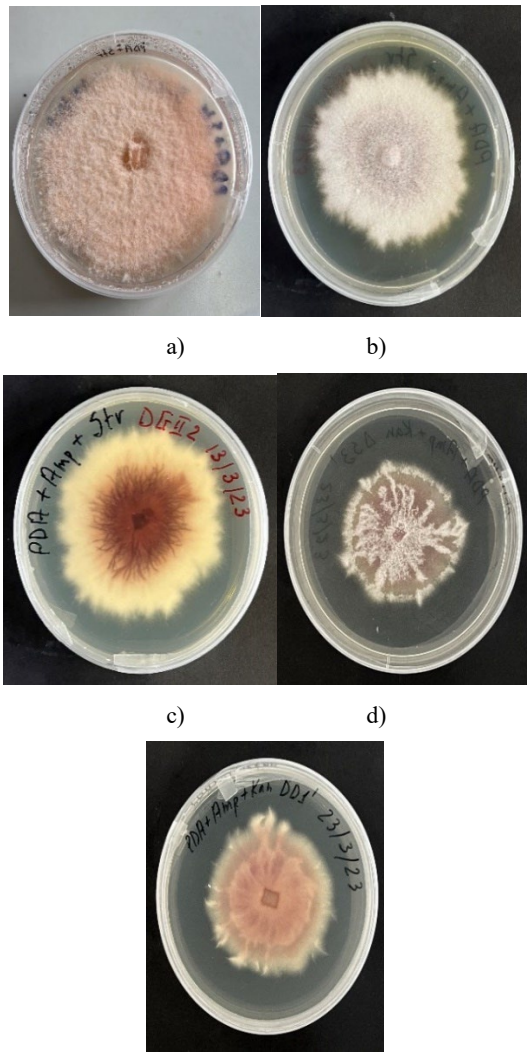


Fig. 1: The main morphological types of colonies isolated on PDA and identified by visual assessment as probably belonging to *Fusarium oxysporum*: a) DG2A; b) DS2; c) DG2; d) DG3; f) DD1'.
Source: compiled by the authors.

Despite the significant morphological heterogeneity inherent in this genus of fungi as a whole, the growth, development and appearance of these colonies had several distinct common features. The first is the delicate morphology of the mycelium and its light colours, varying from yellowish or greyish white to various shades of creamy pink, creamy yellow or translucent coffee. This is a feature that can be identified at any stage of colony development. Another static characteristic inherent in all the morphological types considered is the colony edge – all the fungal colonies sampled and examined have an uneven fibrous edge. All samples also tend to have a rhizoidal colony shape with a clearly defined centre at the point of inoculation.

As for dynamic indicators, a common feature of the samples was the darkening of the mycelium over time. Colonies that were significantly pigmented in the mature

state also developed without pigmentation or with minimal pigmentation for some time at the very beginning of growth. The new hyphae are also mostly less pigmented in all colonies. The centre of the darkening coincides with the centre of the colony and is tied to the point of inoculation. It spreads radially and follows the growth vectors of the hyphae. On most colonies, clear lines, similar to analogues of annual rings, can be observed, separating areas with different pigmentation and may coincide with the number of periods of different mycelial growth intensity. There are usually two or three such distinct areas. The growth of colonies is radial, occurring from the edges, while the central part moves earlier to the state of maturity, sporulation and ageing, and finally to death. On some cups, authors observed the "branching" of daughter colonies from the main one. This did not correlate with specific morphotypes or strains and was largely limited to the small diameter of the Petri dishes.

The sizes of colonies with different morphological types were comparable and ranged from 26 to 38 mm after 7 days of growth. The maximum and minimum colony size, however, correlated with their morphology. The radii of colonies A and C were larger, and D and F smaller, while colonies of morphotype B reached the average size, as illustrated in Figure 1, which shows Petri dishes with the most representative colonies in terms of size and shape. After 6-7 days, the growth of all colonies stopped, and then, until the tenth day, the growth radius did not change or increase by 1 or 2 mm. Before isolating and analysing the genetic material, authors described and analysed the cultural properties of fungal colonies of different morphological types shown in Figure 1.

Colonies of morphological type A reached the largest sizes: from 29 to 38 mm. The mycelium is creamy pink in colour, without darkening after the beginning of fruiting bodies ripening. The fruiting bodies protruded above the mycelium, pigmented similarly to vegetative hyphae. On some cups, pigment was observed to be released into potato agar around the perimeter of the colony. The shape of the colony is round, with a rhizoid edge. The inoculation point is visible, also round and darker than the rest of the mycelium. Hyphae at the inoculation point are denser. The growth rate was determined to be high; the first hyphae were white and loosely arranged, and the growth pattern was adherent, i.e. with incremental growth. Two phases of growth activity were observed. The cessation of growth (growth of less than 0.5 mm) was observed after 8 days, and the beginning of sporulation was recorded on days 5-6. Sporulation was identified as good: 100 or more spores and 10 conidia per microscopic field, at a magnification of 100 and using immersion oil. This was the second most common morphotype among the experimental samples.

Colonies of variant B were the most frequent among the isolates. Their sizes were consistently smaller than those of the previous morphotype and ranged from 23 to 32 mm in diameter. The colour of the mycelium was greyish-white, yellowish at the edges of the colony with the release of yellow pigment into the agar[20),21)]. Colonies are rounded, with an irregular rhizoid edge. The structure of the colony is fibrous. The point of inoculation is visible, it is lighter and more pubescent than the rest of the colony surface. Growth is fluffy, with the active phase occurring twice with a delay period, which is evident in the structure of the colony surface, particularly the presence of a denser ring corresponding to slower growth. The growth rate was determined as average. Growth stopped after 6-7 days, and sporulation began 1 or 2 days earlier. From 11 to 50 spores were recorded in the microscope field at a hundredfold immersion magnification, which corresponds to sporulation of medium activity.

Colonies of sample C were the second largest and third most abundant. The size of these colonies ranged from 28 to 34 mm. The colour of mycelium depends on its age. Young hyphae are creamy yellow and more pubescent, and as the cells age, they begin to synthesise brown pigment unevenly, radially diverging along the growth vectors. The inoculation point is rectangular in shape and is most intensely coloured. Hyphae at the inoculation point are more densely arranged. The colonies are rounded, with an uneven rhizoid margin, and the hyphae are glabrous. The growth pattern is of the adherent type, the growth is uniform. The first hyphae are light, with a slight yellowish tint. Growth cessation was observed on the 7th to 8th day, and the beginning of sporulation in some cases began quite late on the 5th to 7th day. Sporulation is of medium activity: 20 to 51 spores per field of view.

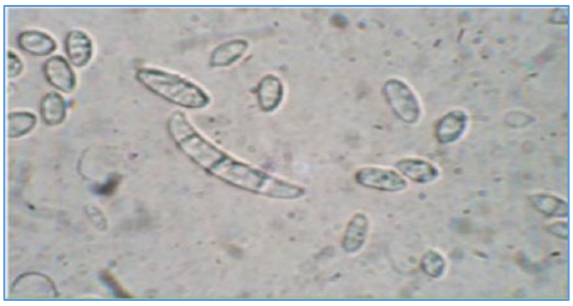
One of the most common, but the smallest in size, were colonies of type D. The diameter of these colonies ranged from 19 to 24 mm. The main mycelium is cocoa-coloured, with pubescent white hyphae bearing conidia. The point of inoculation is rectangular, almost indistinguishable in colour, but limited to sexual hyphae. Sexual hyphae dichotomously and radially diverge from the centre, leaving free space for vegetative mycelium, and are located along the perimeter of the colony. The shape of the colonies is round, with a weakly dissected rhizoid margin. The growth rate is slow, its character is adherent. The growth of colonies of this morphotype stopped the fastest among all samples – in 4 or 5 days, spore formation began in the same period or a day earlier, also the fastest among all samples. Spore production is low to medium: approximately 5 to 20 spores per field of view.

The last morphotype, type F, was much less common than the others. The size of colonies was in the range of 20-25 mm, approximately at the same level as morphotype D. The mycelium is creamy pink in colour, with darker and lighter zones alternating radially. The inoculation point is rectangular, darker than the main part of the mycelium. Colonies are rounded, with an uneven rhizoid margin, and hyphae are slimy. The growth type is adherent, initially fast, then slowing down and continuing at an average rate. Hyphae of the second growth stage are lighter, translucent and almost not pigmented. Complete

cessation of growth occurred on the 6th to 8th day and the beginning of sporulation on the 5-6th day. Sporulation is low – up to 10 spores in the field of view of a hundredfold magnification light microscope.

Macroscopic and microscopic features of representative colonies were determined at 400x magnification and 1000x magnification using immersion oil in the case of chlamydospores. Colonies with microscopic features characteristic of the genus *Fusarium* were allowed for further analysis: with phallate and triseparated macroconidia (24 to 43.8 µm long) and microconidia with zero or one septum (6.8 to 10.4 µm, oval to cylindrical) (Figure 2).

a)



b)

Fig. 2: Images of the fungal strain isolated from the symptomatic part of plants: a) micro- and macroconidia (400x magnification); b) chlamydospores (1000x magnification). Source: compiled by the authors.

The preliminary identification, based on isolation and observation of static and dynamic colony parameters, suggested that *Fusarium oxysporum* is the main cause of wilt symptoms in tomatoes grown in protected ground[22]. Isolates of presumably this species of pathogenic fungi that passed the two-stage verification of morphological, cultural and microscopic parameters were sent for further molecular identification by a polymerase chain reaction to accurately confirm the species and exclude the possibility of error due to the high variability of these parameters in *Fusarium oxysporum*. PCR results provided the following nucleotide sequences for seven samples of five morphotypes of colony isolates:

1. Isolate DD1 Sequence:

TGTGCCCAAACCCCTGTGAACATACCAATTGTT
GCCTCGGCGGATCAGCCCGCTCCCGGTAAAACG
GGACGGCCCGCCAGAGGACCCCTAAACTCTGTT
TCTATATGTAACTTCTGAGTAAAACCATAAATAAA
TCAAAACTTTCAACAACGGATCTCTTGGTTCTGG
GCATCGATGAAGAACGCAGCAAAATGCGATAAG
TAATGTGAATTGCAGAATTCAGTGAATCATCGAA
TCTTTGAACGCACATTGCGCCCGCCAGTATTCTG
GCGGGCATGCCTGTTCGAGCGTCATTTCAACCCT
CAAGCCCCCGGGTTTGGTGTTGGGGATCGGCGA
GCCCTTGCGGCAAGCCGGCCCCGAAATCTAGTG
GCGGTCTCGCTGCAGCTTCCATTGCGTAGTAGTA
AAACCCTCGCAACTGGTACGCGGCGGGGCCAAG
CCGTTAAACCCCCAACTTCTGAATGTTGACCTCG
GATCAGG.

2. Isolate DG2A Sequence:

AGGGGAGGGATCATTACCGAGTTTACAACTCC
CAAACCCCTGTGAACATACCACTTGTTGCCTCGG
CGGATCAGCCCGCTCCCGGTAAAACGGGACGGC
CCGCCAGAGGACCCCTAAACTCTGTTTCTATATG
TAACTTCTGAGTAAAACCATAAATAAATCAAAAC
TTTCAACAACGGATCTCTTGGTTCCTGGCATCGA
TGAAGAACGCAGCAAAATGCGATAAGTAATGTG
AATTGCAGAATTCAGTGAATCATCGAATCTTTGA
ACGCACATTGCGCCTGCCAGTATTCTGGCGGGCA
TGCCTGTTCGAGCGTCATTTCAACCCTCAAGCAC
AGCTTGGTGTTGGGACTCGCGTTAATTCGCGTTC
CCCAAATTGATTGGCGGTCACGTCGAGCTTCCAT
AGCGTAGTAGTAAAACCCTCGTTACTGGTAATCG
TCGCGGCCACGCCGTTAAACCCCAACTTCTGAAT
GTTGACCTCGGATCAGGTAGGAATACCCGCTGAA
CTTAAGCATATCAATAG.

3. Isolate DG3 Sequence:

GGACATTACCGAGTTTACAACTCCCAAACCCC
TGTGAACATACCACTTGTTGCCTCGGCGGATCAG
CCCGCTCCCGGTAAAACGGGACGGCCCGCCAGA
GGACCCCTAAACTCTGTTTCTATATGTAACTTCTG
AGTAAAACCATAAATAAATCAAAACTTTCAACAA
CGGATCTCTTGGTTCTGGCATCGATGAAGAACGC
AGCAAAATGCGATAAGTAATGTGAATTGCAGAAT
TCAGTGAATCATCGAATCTTTGAACGCACATTGC
GCCCGCCAGTATTCTGGGGGGCATG.

4. Isolate DS2 Sequence:

ACCCCCACACCCCTGTGAACATACCACTTGTT
GCCTCGGCGGATCAGCCCGCTCCCGGTAAAACG
GGACGGCCCGCCAGAGGACCCCTAAACTCTGTT
TCTATATGTAACTTCTGAGTAAAACCATAAATAAA
TCAAAACTTTCAACAACGGATCTCTTGGTTCTGG
CATCGATGAAGAACGCAGCAAAATGCGATAAGT
AATGTGAATTGCAGAATTCAGTGAATCATCGAAT
CTTTGAACGCACATTGCGCCCGCCAGTATTCTGG
CGGGCATGCCTGTTCGAGCGTCATTTCAACCCTC
AAGCACAGCTTGGTGTTGGGACTCGCGTTAATTC
GCGTTCCTCAAATTGATTGGCGGTCACGTCGAGC
TTCCATAGCGTAGTAGTAAAACCCTCGTTACTGG
TAATCGTCGCGGCCACGCCGTTAAACCCCAACTT
CTGAATGTTGACCTCGGATCAGGTAGGAATACCC
GCTGAACTTAAG.

5. Isolate DG2A Sequence:

GGAAAAAGAACCATTCGATTTCACCTACGACT

CGAAACGTGCCCGCTACCCCGCTCGAGACCAAA
AATTTTGCAATATGACCGTAATTTTTTTTGGTGG
GGCACTTACCCCGCCACTTGAGCGACGGGAGCG
TTTGCCCTCTTAACCATTCTCACAACCTCAATCA
GTGCGTCGTCACGTGTCAAGCAGTCACTAACCAT
TCAACAATAGGAAGCCGCTGAGCTCGGTAAGGG
CTCCCT.

4. Isolate DG3 Sequence:

CGATTTCCCCTACGACTCGAAACGTGCCCGCT
ACCCCGCTCGAGACCAAAAATTTTGCAATATGAC
TGTAATTTTTTTTGGTGGGGCACTTACCCCGCCA
CTTGAGCGACGGGAGCGTTTGCCCTCTTAACCAT
TCTCACAACCTCAATGAGTGCGTCGTCACGTGTC
AAGTAGTCACTAACCATTCAACAATAGGAAGCCG
CTGAGCTCGGTAAGGGTC.

5. Isolate DS2 Sequence:

CTTCAATACCTACGACTCGAAACGTGCCCGCT
ACCCCGCTCGAGACCAAAAATTTTGCAATATGAC
CGTAATTTTTTTTGGTGGGGCACTTACCCCGCCA
CTTGAGCGACGGGAGCGTTTGCCCTCTTAACCAT
TCTCACAACCTCAATGAGTGCGTCGTCACGTGTC
AAGCAGTCACTAACCATTCAACAATAGGAAGCC
GCTCGAGCTCGGTAAGGGTCCCAT.

For morphotypes DS2, DG2A and DG3, nucleotide sequences were determined by polymerase chain reaction twice, using extracted genetic material from isolates of different colonies and using different primers. The use of different primers, respectively, led to the difference between the sequencing products of the same morphotype and the fact that the obtained fragments were of different sizes.

Comparison of the obtained sequences with those stored in the nucleotide sequence database of the National Centre for Biotechnology Information (NCBI) GenBank using the BLASTn tool allowed to obtain the final identification results. Thus, for the isolates of samples DG2A, DG3, DS2 and DG2, their species affiliation to *Fusarium oxysporum* was confirmed by all sequences. At the same time, the nucleotide sequence of the sample of morphological type DD1, according to the results obtained from the database search, belonged to another species of the genus *Fusarium* – *Fusarium proliferatum*.

The study shows that different environmental variables have a substantial impact on the formation and dissemination of *Fusarium oxysporum* morphotypes. Root endophytes had a higher diversity than stem endophytes, indicating that root conditions are more favorable for the development and spread of a wide variety of *Fusarium* morphotypes. *Fusarium* colonies became less diverse in quantity as the distance from the root increased[23]. The variety of fungal species was strongly impacted by the intensity of the disease symptoms, as *Fusarium oxysporum* grows best in conditions where the host plant is already compromised by the illness. It was discovered that individual plants and farms had a greater influence on morphological diversity within *Fusarium* colonies than tissue location. It was possible to identify five primary morphological forms of *Fusarium oxysporum* colonies, each with unique traits. The results highlight the significance of taking local factors, such as soil type, moisture content, and farming techniques, into account when researching *Fusarium oxysporum* epidemiology, as these factors have a major influence on the spread and growth of various *Fusarium* morphotypes.

The pathogen that causes *Fusarium* wilt in tomatoes, *Fusarium oxysporum* f. sp. *lycopersici*, may be efficiently controlled using a mix of biological, chemical, and agrotechnical techniques. Crop rotation is one agrotechnical technique that helps prevent the spread of pathogens by planting non-host crops in the same soil for many growing seasons[24]. In many vegetable crops, including tomatoes, soil solarization, which entails covering the soil with sheets of transparent polyethylene for four to six weeks during the warmest portion of the year, has been demonstrated to lower the incidence of *Fusarium* wilt[25].

Fungicides such as fludioxonil or thiophanate-methyl, which may be employed as part of an integrated management approach, are among the chemical methods. *Fusarium* populations in the soil can be successfully decreased by using soil fumigants, which are usually used before to planting in order to sterilise the soil. However, because of their toxicity and potential effects on the environment, they must be handled carefully and safety standards must be followed.

By using helpful microbes, such as Trichoderma species, which may outcompete *Fusarium* for nutrients and space, create antifungal chemicals, and increase plant tolerance, biological approaches can decrease *Fusarium oxysporu*m. *Pseudomonas fluorescens* and *Bacillus subtilis* are examples of beneficial bacteria that have been employed as biocontrol agents to prevent *Fusarium* wilt.

Another successful biological control tactic is the development and planting of resistant varieties through breeding programmes[26]. The best way to reduce *Fusarium* wilt and improve tomato crop production and health while reducing environmental impact is to combine these techniques into an integrated pest management (IPM) strategy.

Thus, despite the effectiveness of culture and microscopic identification, the results obtained from PCR slightly adjusted the previous assumptions. However, it was confirmed that in all plants with visible signs of the disease affecting the root and lower part of the stem, the infected tissues contained *Fusarium oxysporum* hyphae.

4. Discussion

Fusarium or tomato wilt is a common plant disease caused by endophytic parasitic fungi. Many environmental variables affect the morphological changes of *Fusarium oxysporum*. The morphology of the pathogen is influenced by a number of factors, including temperature, humidity, moisture content, availability of nutrients, agricultural methods, and microbial

interactions[27]. Larger colonies are supported by soils rich in organic matter; smaller, less robust colonies are produced by soils deficient in nutrients. Variations in temperature have an impact on the development and coloration of fungi, and different microenvironments are created by farming techniques and regional factors. Morphology is also influenced by interactions with other microorganisms in the rhizosphere of plants and soil. It is essential to comprehend these elements in order to create management plans that effectively combat *Fusarium* wilt.

In closed-ground conditions, when plants are either isolated from other plants of their own or other species, or their contacts are severely limited or indirect, the likelihood of disease occurrence among them is significantly reduced. The possibility of exogenous inoculation with pathogens can be minimised by quarantine and the use of pesticides of varying specificity to reduce the number of weeds and insects that could potentially become vectors[28]. Therefore, endophytic parasitic fungi and bacteria pose a major threat to the growth, development and productivity of crops[29),30].

In this study, 50 tomato plants were selected among greenhouse plants that showed signs of wilt: wilting of leaves, necrotic spots near the vascular fibre bundles and on the stems. *Fusarium oxysporum* was detected in the affected tissues of each of these plants, and in some morphologically similar *Fusarium proliferatum* was found. A similar study on the isolation and identification of tomato wilt pathogens was conducted by L. Murugan et al.[31]. The cultural analysis was confirmed by determining the nucleotide sequence by polymerase chain reaction. As a result, the authors concluded that all affected tissues contained strains of *Fusarium oxysporum* f. sp. *lycopersici*. Among these strains, three different morphological types were recorded, which is one less than in the present study, but these results also indicate that strains with different cultural and morphological properties cause the same symptoms in plants. Both studies confirmed *Fusarium oxysporum* f. sp. *lycopersici* in tomatoes and used molecular techniques for identification. The current study focused on race identification, while Murugan et al. analysed different races, highlighting genetic diversity and disease management implications.

A similar study was also carried out by K. Vignesh et al. at the Colombian Research Institute. The subject of the study was the collection of strains of *Fusarium oxysporum* and the affected tomato plants themselves[13]. Isolation and identification were performed in the same way. Phylogenetic analysis was performed using EF1a. The authors report that most of the strains isolated from the affected tissues belonged to *Fusarium oxysporum* and specifically *Fusarium oxysporum* f. sp. *lycopersici*. A strain of *Fusarium solani* was indirectly isolated. This study also noted that different morphotypes of fungi cause the same manifestations. Both studies highlighted the widespread presence of *Fusarium oxysporum* f. sp. *lycopersici* and its severe impact on tomato crops. However, the current study reveals that root endophytes exhibit greater diversity than stem endophytes, and *Fusarium* colony diversity decreases with distance from the root. This differs from the study by Vignesh et al., which focused on the geographical distribution and prevalence of *Fusarium* wilt in tomato-growing areas of Krishnagiri district, India, rather than the microbial ecology within individual plants.

Additionally, the issue of identification and study of genetic diversity of endophytic pathogenic fungi is relevant for Q. Ye et al.[32]. They note that in recent years, cases of massive damage to tomato plants by root rot and wilt have become more common in China. This trend has affected tomatoes resistant to different morphotypes of the most common and already mentioned strain of *Fusarium oxysporum* f. sp. *lycopersici*. When sampling tissues of affected plants and isolating pathogen isolates, more than 70% of different strains of *Fusarium oxysporum* were obtained, most of which showed high virulence. Here, the authors noted that different morphotypes of isolates showed different specificity for plant infection, including resistant varieties. *Fusarium proliferatum* and *Fusarium solani* strains also accounted for a significant proportion of the isolates. Research in China by Ye et al. focused on the genetic diversity of wilt and root rot pathogens in tomatoes, using both morphological and molecular methods. The latter research, on the other hand, focused on *Fusarium oxysporum* in a specific region of Albania, providing a more comprehensive view of the pathogen landscape.

Two more studies by C.M. de Oliveira et al. describe the processes of diagnostics among affected tomatoes of local farmers in Brazil and Mexico, respectively[33]. The authors of both papers used similar principles: they sampled affected stem and root tissues, isolated colonies and implemented a two-step identification based on cultural and morphological characteristics, which were supported by microscopy in the first stage and by polymerase chain reaction in the second stage. The authors identified 63 and 96% of the isolates as belonging to recognised strains of *Fusarium oxysporum*, and all the others were identified as *Fusarium spp*. The authors from Brazil noted the difficulties of identification arising at the stage of determining the nucleotide sequence of atypical fungal strains they pointed out that primers were not always suitable for the two morphotypes of *Fusarium oxysporum*. The study conducted in Mexico was notable for the fact that visual assessment of plants for pathogenic *Fusarium* fungi was effective for only 75% of the samples, while the rest did not show any disease caused by pathogens. However, both studies noted PCR as an effective and convenient method for diagnosing *Fusarium* pathogens[34].

Of interest in this context is the work of R. Dhaya, in which the author presents her algorithm for diagnosing *Fusarium*[35]. The author refers to the traditional method of visual assessment of this disease based on the appearance of tomato leaves and offers a software option with a built-

in version of artificial intelligence, which was trained based on 87 thousand images of healthy and affected leaves. The diagnostic accuracy presented in the paper is 96%. The visual assessment in this study was 100% effective, as all tomato tissues sampled in the Berat and Lushny greenhouses were found to be affected by wilt pathogens. Thus, in general, in domestic conditions, the method of visual assessment, manual or automated, is quite effective and acceptable.

To prepare a site for growing plants in an indoor environment, it is advisable to prepare the site and examine the soil microflora, both on the site and in the imported soil. Preventive detection of pathogens, respectively, can protect the cultivated crops both from the pathogenic strains themselves and from the residues of products that can be used to eliminate these undesirable organisms. The work by D. Jiménez-Fernández et al. studied the possibility of isolating and identifying *Fusarium oxysporum* strains directly from soil using the capabilities of the Real-Time Polymerase Chain Reaction[36]. The authors were able to effectively diagnose this parasitic fungus in both soil and chickpea roots. They noted that foreign nucleic acids present in the medium did not affect the accuracy of the diagnosis. Accordingly, they developed a diagnostic protocol that, depending on the purpose and convenience of the farm, can be used both for soil analysis and for checking the presence of the target fungus in the roots of plants growing in this medium.

The issue of virulence of *Fusarium oxysporum* strains using protein analysis was investigated by a group of scientists led by R. Manikandan et al., they chose the most common strain of *Fusarium oxysporum* f. sp. *lycopersici*[37]. In the course of the work, proteomics approaches were used to grade 20 isolates according to their increasing virulence. Virulence was higher the greater the number of corresponding bilayers synthesised by the isolates. Reducing virulence may be one way to combat this pathogen. Another alternative and environmentally friendly way is to look for natural enemies. Their study offers insight into the molecular processes that underlie the pathogen's capacity to infect and damage tomato plants. In contrast to the morphological diversity noted in the current study, this proteomic analysis provides a distinct viewpoint on pathogen variability. The observations made by the latter research on the different colony features and development patterns among the isolates of *Fusarium oxysporum* are consistent with the proteome differences reported by Manikandan et al. Despite approaching the subject from distinct angles, morphological and proteomic, both studies demonstrate the variety found in *Fusarium oxysporum* populations.

Recently, in 2022, authors from China, led by J. Wang et al., discovered and described the first coronavirus capable of infecting *Fusarium oxysporum* mycelial cells[38]. According to the authors, this virus is species-specific and does not affect other species of the *Fusarium* genus. Infection with this virus causes a delay in the growth and development of mycelium, as well as the formation of fruiting bodies, reduces the number and deteriorates the quality of spores. At the same time, no significant decrease in virulence was recorded[39)-42)].

In general, there are many relevant studies in recent years devoted to the study of tomato wilt, isolation and identification of its pathogens. The results of most of the authors are comparable to the present study and indicate the effectiveness of PCR and morphological and cultural methods in the detection of *Fusarium oxysporum*. Also, in the absence of the need for a highly specific response, a visual assessment of diseased plants may be sufficient to diagnose diseases caused by this fungus[43)-45)].

Despite the fact that the research's methodologies offer insightful information regarding the identification and characterization of *Fusarium oxysporum* f. sp. *lycopersici*, there are a number of limitations and possible causes of inaccuracy. These include the subjective nature of morphological judgements and technological problems with DNA identification. The selection of symptomatic plants from specific regions (Berat and Lushnja) may not fully represent the diversity of *Fusarium oxysporum* f. sp. *lycopersici* present in other tomato-growing areas. Also, it should be noted that microscopic identification may be inaccurate due to the similar morphological characteristics of different species. The accuracy and thoroughness of pathogen identification and characterization efforts would be improved by addressing these constraints in further research.

5. Conclusions

In protected ground, the greatest danger to plants is caused by diseases of endophytic microorganism origin. In the cultivation of tomatoes (*Solanum lycopersicum L.*), such endophytes are microscopic fungi *Fusarium oxysporum*. Given the considerable variability in morphological and cultural characteristics of strains of this species, its identification by standard methods can be difficult. Therefore, to accurately identify the pathogen, this study proposed to confirm the preliminary results obtained by visual assessment of damage and traditional microbiological methods by using the possibility of determining the nucleotide sequence by PCR and its subsequent comparison with sequences collected in the GenBank of the National Center for Biotechnology Information using BLASTn search.

The preliminary visual identification of the affected plants was successful – all the tissues sampled contained *Fusarium oxysporum* cells. This also confirmed that these fungi are the main cause of tomato diseases. At the same time, root tissue contained more biodiversity than stem tissue. Initially, 5 morphological types of colonies were identified as likely to belong to *Fusarium oxysporum* based on cultural characteristics and light microscopy. However, the analysis and comparison of nucleotide sequences showed that one of the selected morphotypes

belonged to another fungal species, *Fusarium proliferatum*. At the same time, there was no clear correlation between the morphotype of the fungus and the intensity of the disease. This topic requires more attention in future studies.

Thus, morphological and cultural identification in combination with light microscopy has proven to be a good method that can be used in conditions that do not require absolute accuracy. In studies where species identification is a critical or target indicator, it is advisable to use methods that include the detection and analysis of nucleotide sequences. In small farms, when choosing a non-specific treatment or prevention of *Fusarium*, a visual assessment of the symptomatic manifestations of the disease on tomato leaves and stems may be sufficient. Further research could also be aimed at studying the virulence of different types of *Fusarium oxysporum* strains, or at developing a specific treatment using viruses specific to this species.

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