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Vegetative Growth and Molecular Identification of *Fusarium equiseti* Isolated from Wilt Disease of *Centella asiatica* L. in Bangladesh

Poli Akter[1], Shahida Khatun[1], Durga Das Bhowmik[1], Farzana Ashrafi Neela[2], Nuhu Alam[1*]

[1]Laboratory of Mycology and Plant Pathology, Department of Botany, Jahangirnagar University, Savar, Bangladesh
[2]Department of Botany, University of Rajshahi, Rajshahi, Bangladesh
Email: *mnabotju@juniv.edu

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Abstract

Centella asiatica (L.) is commonly known as Thankuni plant and has ethnobotanical importance in Bangladesh. Present experiment was conceded to investigate the wilt disease of *C. asiatica*, vegetative growth and molecular characterization of pathogenic fungi. Pathogenic fungus, *Fusarium equiseti* was identified as a causal agent of wilt disease in *C. asiatica*. The effect of culture media on the mycelial growth of *F. equiseti* showed the highest (89.25 mm) on potato dextrose agar (PDA) medium followed by carrot agar (CA) medium and the lowest growth (40.25 mm) was measured in HA medium. The optimal temperature and pH for mycelial growth of *F. equiseti* were 30˚C and 7, respectively. The genetic variation of the selected species of fungi, the internal transcribed spacer (ITS) region was amplified using ITS4 and ITS5 primers and sequenced. The PCR product of the ITS region of *F. equiseti* was 535 bp. Phylogenetic tree of thirty-seven strains of *Fusarium* sp. based on the nucleotide sequences of the ITS region using the neighbor-joining method with 1000 bootstrapping indicated that 98% - 100% identity with MN886590.1 JUF0046 (*F. equiseti*). ITS sequences are generally constant, or show little variation within species, but vary between species in a genus. The ITS region is relatively short and can be easily amplified by PCR using universal single primer pairs. Genetic distance exhibited high level of similarity with identical ITS sequences. To date, no published research articles are found on the molecular identification of *F. equiseti*, the causal agent of fusarium wilt disease of *C. asiatica* in Bangladesh.

Keywords

Centella asiatica (L.), *Fusarium equiseti*, Molecular Identification, Vegetative Growth, Wilt Disease

1. Introduction

Centella asiatica (L.) is a prostrate, faintly aromatic, stoloniferous, perennial, creeper herb, commonly known as Thankuni in Bengali [1]. It is found almost all over the world and flourishes extensively in shady, marshy, damp and wet places such as paddy fields, river banks forming a dense green carpet. The leaves, which are edible, are in yellowish-green color, thin, alternate with long petioles, and quite characteristic reniform, orbicular, or oblong-elliptic shapes with seven veins. The plant grows horizontally through its green to red stolones which combine with each other and roots underground [2].

It is an important plant with wide range of traditional, medicinal and therapeutic values and is utilized as a source of food, beverages, medicine. This is a key element of numerous compound formulations used for curing gastrointestinal disorders, cutaneous problems and for revitalizing brain cells. In Southeast Asia, it is traditionally used for the treatment of a wide variety of disorders such as skin diseases, rheumatism, inflammation, syphilis, mental illness, epilepsy, hysteria, dehydration, and diarrhea [1] [3]. The chemical constituents of *C. asiatica* have a very important role in medicinal and nutraceutical applications and it is believed due to its biologically active components of triterpenes saponins. The triterpenes of *Centella* are composed of many compounds including asiatic acid, madecassic acid, asiaticosside, madecassoside, brahmoside, Brahmic acid, brahminoside, thankiniside, isothankunisode, centelloside, madasiatic acid, centic acid, and cenellicacid. Due to their importance, they have been used as the biomarker components for quality assessment of *Centella* [4]. According to Zainol *et al.* [5] the highest concentration of phytochemicals was found in the leaves relative to the petioles and the roots. It is also rich in vitamin C, vitamin B1, vitamin B2, vitamin A and niacin carotene.

The demand for *C. asiatica* in different cases especially for medicinal purpose is increasing day by day. Nowadays, it is cultivated in different parts of the world. But the major problem in its cultivation is different diseases caused by microorganisms, especially by fungi. The diversity of the endophytic fungi was classified as a medium diversity. The highest diversity of the endophytic fungi was obtained from the roots, followed by the leaf, stolon, and petiole. *Ceratobasidium* sp., *Colletotrichum* sp., *C. destructivum*, *Fusarium solani*, *Alternaria* and *Curvularia*, were the most dominant endophytic fungi in plant organs of *C. asiatica* in Malaysia. Souza and Santos [6] reported that *Colletotrichum* spp. and *Fusarium* spp. were the most common endophyte fungi that cause major diseases in various medicinal plants. *Fusarium* sp. is associated with wilt disease of *C. asiatica* which is characterized by browning of the leaf and dark brown lesion development on leaf surface finally the leaf become dry out.

Assessment of genotypic and phenotypic diversity is helpful to distinguish of pathogenic fungi. Different genetic tools have been introduced such as ITS sequence, restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD) and small subunit rhibosomal DNA (rDNA).

Among these tools, the ITS region of genomic DNA is more useful for the identification of pathogenic fungi. The ITS of rDNA is considered as a variable regiom among the species and even among the strains [7] [8] [9]. Many published research articles are available the fungal diseases of many medicinal plants but until up to date, no articles are found in Bangladesh on *C. asiatica* diseases and their management. So considering the pharmaceuticals, therapeutic and other important uses of *C. asiatica*, this research will provide valuable information the extensive cultivation of *C. asiatica* in Bangladesh and appropriate management of wilt disease caused by *F. equiseti* associated with this important medicinal herb.

2. Objectives

The objectives of this study was undertaken deal with the accurate identification of wilt disease caused by *Fusarium equiseti* associated with *Centella asiatica* based on morphological, growth characteristics and molecular approach.

3. Materials and Methods

3.1. Sample Collection

Wilt disease of *Centella asiatica* was collected from different areas of Jahangirnagar University campus. Collected diseased samples were separately packed in sterile polyethylene bags to avoid secondary infection. These samples then kept in the Laboratory of Mycology and Plant Pathology, Jahangirnagar University, Savar, Dhaka for further investigations.

3.2. Fungus Isolation and Morphological Characterization

Fungus was isolated through tissue planting method. Infected parts of *C. asiatica* were cut in to small piece about 0.5 cm in length in such a manner so as to include both fungal infected and non-infected tissues in piece. Then sterilization was done using NaOCl (5%) solution for 5 minutes and rinsed with distilled water several times. Four pieces of samples were placed into PDA medium and were incubated under 12/12 hours dark and light condition at 25˚C ± 2˚C for 10 days. Mycelial growth of growing fungus colony was transferred to fresh PDA plates as well as PDA slants to obtain a pure culture. The pure culture of the isolated fungus was identified microscopically using standard methods based on colony morphology, mycelium and conidia [10].

3.3. Effect of Culture Media, Temperature and pH

Eight different culture media namely, sucrose glucose agar (SGA), yeast extract agar (YEA), honey peptone agar (HPA), maltose agar (MA), carrot agar (CA), potato dextrose agar (PDA), honey agar (HA) and potato sucrose agar (PSA) were used to assay the mycelial growth of the pathogen according to Sultana *et al.* [11]. The media will be adjusted to pH 6.5 before autoclave. Different temperatures (15˚C, 20˚C, 25˚C, 30˚C and 35˚C) were maintained for the mycelial

growth of the pathogen on PDA in an incubator. The mycelial growth was recorded at 7 days post inoculation (dpi) respectively [8]. Different pH levels *viz.*, 5.0, 6.0, 7.0, 8.0 and 9.0 were used [9]. The medium was adjusted to pH 4, 5, 6, 7 and 8 with the addition of 1 N NaOH or HCl before autoclave and it will be incubated at 30˚C for 7 days. Radial growth of mycelia on each Petri dish was measured at 3 directions according to Alam *et al.* [8].

3.4. Molecular Characterization

After microscopic examination, molecular characterization was done with the help of commercial service provided by Invent Technology, Dhaka, Bangladesh. Fungus genomic DNA samples were extracted using the Maxwell Cell Kit (AS1030, Promega, USA). The primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAA AGTCG TAACAAGG-3') were used for the PCR reaction [7]. The PCR reaction was performed with 20 ng of genomic DNA as the template in a 25-µL reaction mixture having a LA Taq (TAKARA BIO INC, Japan). The thermal cycle was performed with activation of Taq polymerase at 94˚C for 1 minute; 35 cycles of 94˚C for 30 Sec, 55˚C for 30 Sec, and 72˚C for 5 minutes each; finishing with a 10-minute step at 72˚C for final extension. Amplified PCR products were electrophoresed on 1.5% agarose gel in 1 × TAE buffer for 1 hr at 100V with a 1kb DNA ladder as a size marker and then stained while agitated in an EtBr solution (0.5% µg/mL). The stained gels were visualized and photographed using a UV transilluminator (Kodak Image Station 4000R; Molecular imaging system, Carestream Health Inc., 150 Verona Street, Rochester, NY 14608). The Maxwell® 16 DNA Purification Kits was used to purify the amplification products (Promega, USA). The purified PCR products were sequenced bi-directionally in First BASE Laboratories SdnBhd (Malaysia).

DNA sequences were checked by BioEdit and MEGA6. Sequencing data were submitted to the NCBI, under accession number JUF0039. A BLAST search with the ITS sequences were used to reveal the closest matching taxa. Multiple sequence alignments were done using MEGA6. Data was converted from fasta to MEGA format with Clustal W. The models of evolution were determined under the Akaike Information Criterion (AIC). The model selected was Tamura-3 parameter for analysis. Maximum likelihood (ML), Neighbor-joining (NJ), and Maximum parsimony (MP) analysis were done and robustness of the branches were determined with 1000 bootstrap replicates along with max-trees set at 1000. The number of replications was inferred using the stopping criterion. Bootstrap values greater than 60% were accepted [12].

4. Results and Discussion

4.1. Morphological Characteristics of *Fusarium equiseti*

Aerial mycelia were abundant and pinkish-white (**Figure 1(a)**). Pale to violet pigmentation formed in agar and the colony centers often were heavily pigmented. Sporodochia were absent. Microconidia were abundant in false heads or

chains from polyphialides, and less often from monophialides, mostly 5-septate, oval, obovoid with a truncate base, elliptical in shape (Figure 1(b)). Macroconidia were extremely sparse and difficult to find but, where present, they were typically three-septate, fusiform with tapered apical cell and with poorly developed foot-shaped basal cells.

4.2. Effect of Culture Media, Temperature and pH

The effect of culture media *viz.*, SGA, YEA, HPA, MA, CA, PDA, HA and PSA on mycelial growth of *Fusarium equiseti* have been presented in Figure 2 and Figure 3. The results showed that the highest mycelial growth (89.25 mm) of *F. equiseti* was recorded on PDA medium which was followed by CA medium and the lowest growth (40.25 mm) was measured in HA medium.

PDA is one of the most commonly used culture media because of its simple formulation and its ability to support mycelial growth of a wide range of fungi. Several workers stated that PDA to be the best medium for mycelial growth of

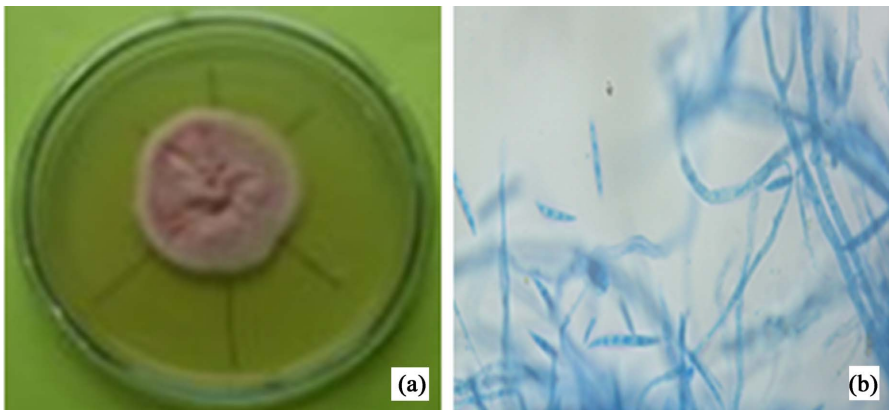
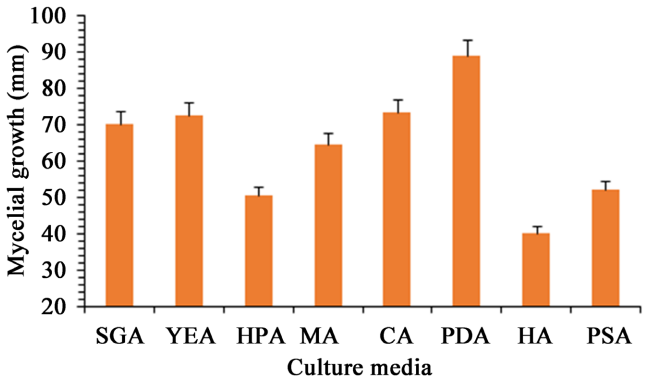
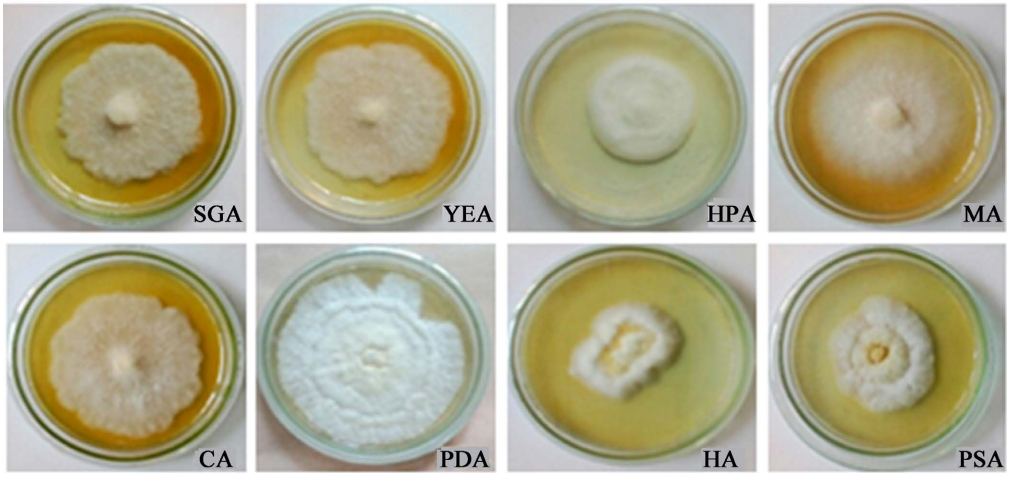


Figure 1. Morphological characteristics of *Fusarium equiseti*. (a) Mycelial growth of *F. equiseti* on PDA medium, (b) Microscopic view of mycelium and conidia of *F. equiseti* (40 × 10×).



SGA, sucrose glucose agar; YEA, yeast extract agar; HPA, honey peptone agar; MA, maltose agar; CA, carrot agar; PDA, potato dextrose agar; HA, honey agar; PSA, potato sucrose agar.

Figure 2. Effect of different culture media on the mycelial growth of *Fusarium equiseti* at 7 dpi.



SGA, sucrose glucose agar; YEA, yeast extract agar; HPA, honey peptone agar; MA, maltose agar; CA, carrot agar; PDA, potato dextrose agar; HA, honey agar; PSA, potato sucrose agar.

Figure 3. Effect of different culture media on mycelial growth of *Fusarium equiseti.*

fungi [13] [14]. The present studies confirm the reports of earlier workers doing their physiological studies related to suitable media for growth and sporulation of *Fusarium* sp. Khan *et al.* [15] reported that *Fusarium oxysporum* f.sp. *ciceri* had the highest mycelial growth on PDA (85.76 mm) followed by Richard's medium (84.62 mm) and Czapek's medium (72.56 mm). Pradeep *et al.* [16] cited the maximum radial mycelial growth on PDA (74.96 ± 0.35 mm) followed by Malt extract Agar (74.03 ± 0.25 mm) and Oat Meal Agar (74.03 ± 0.15 mm). Agar medium which is supported by Hadwiger [17] who noted of the 78.6 mm, 75.8 mm and 74.0 mm mycelial growth of *Fusarium solani* in PSA and CA media, respectively. So this study has revealed the PDA was the best medium for mycelial growth of isolated fungi.

The optimum temperature is important criteria for understanding the ecology of spoilage fungi. Present study investigated the effect of temperature on radial mycelial growth of *Fusarium equiseti* on PDA medium under *in vitro* condition. The experimental plates were incubated at five different temperature viz., 15˚C, 20˚C, 25˚C, 30˚C and 35˚C and the results have been shown in **Figure 4**. The data informed that the highest growth of *F. equiseti* was recorded at 30˚C, followed by 35˚C. In our experiment, *F. equiseti* grew the maximum at 30˚C which is consistent with the previous findings of Iwen *et al.* [18] who cited that the highest mycelia growth and sporulation of *Mucor circinelloides* registered at 30˚C, a sudden fall in mycelial growth and sporulation observed at 30˚C and 35˚C. *M. circinelloides* grew with well-developed mycelium at 25˚C to 35˚C. This showed that temperature has no effect on the growth of the mycelial growth of *F. equiseti*. There is always an optimum temperature for the best growth of any fungus as temperature has a regulatory effect on fungal growth and development. Daami-Remadi *et al.* [19] observed that thermal optimum of 30˚C was suitable for maximum mycelial growth of *Fusarium solani*. Singh *et al.* [20] noticed that the optimum temperature for growth and sporulation of *Fusarium solani* was 30˚C.

pH is also one of key criteria for understanding the ecology of spoilage fungi, however, in present study, the experimental plates were incubated at five different pH level *viz*., 5, 6, 7, 8 and 9. Maximum mycelia growth (82.25 mm) of *Fusarium equiseti* was recorded at pH 7 and followed by pH 8 and pH 6, while minimum mycelial growth was found at pH 8 and pH 9, respectively (**Figure 5**). Sharma *et al.* [21] studied the effect of pH on the growth of *F. oxysporum* and reported that *Fusarium* sp. could sporulate grew well at 5.5 pH. Gangadhara *et al.* [22] reported that the maximum growth of *Fusarium oxysporum* occurred at pH 5 and pH 6 *in vitro*. Similar results are also reported the earlier who obtained the maximum growth of *Ceratocystis fimbriata* at pH 7.5, followed by pH 7.0 and pH 8.0 [23]. Yadahalli *et al.* [24] noticed the maximum mycelial growth of *Ceratocystis paradoxa* when the pH of the medium was between 6.0 and 7.5. Result indicated that this fungus grew well at neutral condition.

4.3. Molecular Characterization

The internal transcribed spacer regions of fungal rDNA are highly variable sequences of great importance in distinguishing fungal species by PCR analysis.

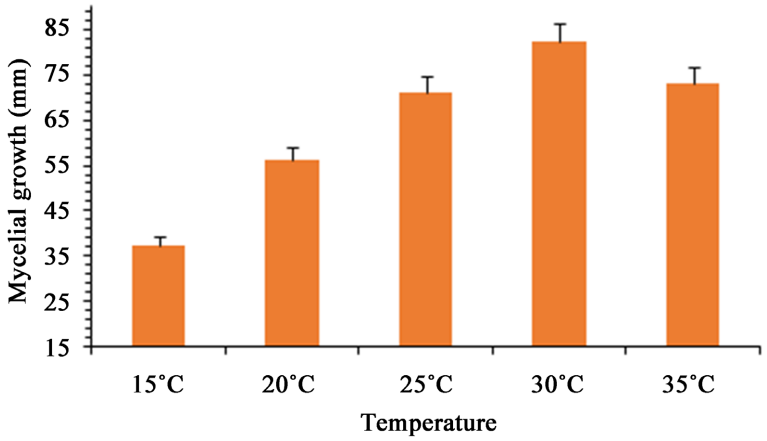


Figure 4. Effect of different temperature on the mycelial growth of *Fusarium equiseti* at 7 dpi.

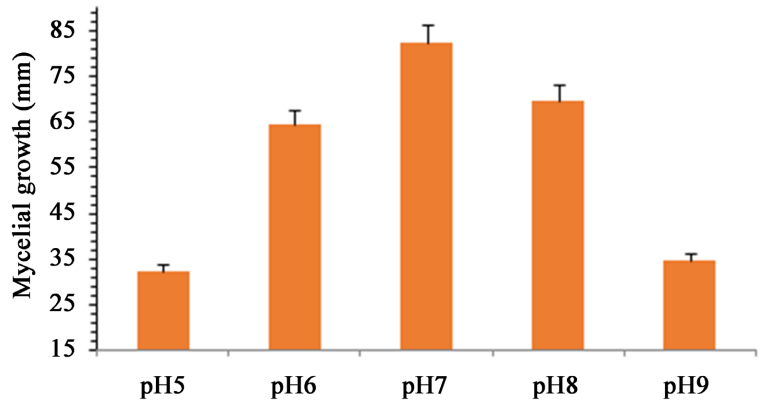


Figure 5. Effect of different temperature on mycelial growth of *Fusarium equiseti* at 7 dpi.

For molecular identification, the ITS region of 535 bp was amplified using ITS4 and ITS5 primers and sequenced. The size of the internal transcribed spacer region as measured by gel electrophoresis of PCR products, amplified by primers ITS4 and ITS5, was 535 bp for *Fusarium equiseti*, but other species of this genus had a shorter ITS region, making this characteristic potentially useful in the identification of *Fusarium equiseti* (**Figure 6**). Recent molecular phylogenetic studies have demonstrated that the ITS region of genomic DNA is very useful for identification of fungi at lower taxonomic levels. The internal transcribed spacer of rDNA is considered as a variable region among the species and even among the strains [8].

The ITS region was amplified using ITS4 and ITS5 primers and sequenced. Phylogenetic tree based on the nucleotide sequences of the ITS regions in thirty six fungal taxa were selected from the NCBI database for phylogenetic analysis. In maximum parsimony tree there are seven different clusters were found in the phylogenetic tree. Per cent homology of rDNA sequence of ITS region (MN886591.1) was compared with formerly identified fungi MN559437.1 *Fusarium equiseti*, MH368109.1 *Fusarium equiseti*, HM008677.1 *Fusarium equiseti*, KX576658.1 *Fusarium equiseti*, KY031974.1 *Fusarium equiseti*, MH128127.1 *Fusarium equiseti* and MK733980.1 *Fusarium equiseti* (**Figure 7**). Reciprocal homologies of the ITS region sequences ranged from 98 to 100%. The sequencing data of the selected NCBI GenBank strain AM261761.1 *Fusarium oxysporum* was used as control for the comparative studies on phylogenetic relationships with the identified fungus of *Fusarium equiseti* (MN886591.1, JUF0043). The results indicated that all the individual species of *Fusarium equiseti* belong to major cluster. The ITS region is relatively short and can be easily amplified by PCR using universal single primer pairs. Genetic distance exhibited high level of similarity with identical ITS sequences. The size variation was caused by differences in the number of nucleotides, revealing that these strains are clearly distinguishable from each other based on the ecological distribution, substitution, and insertion or deletion polymorphisms of the base position [8]. Alam *et al.* [7] reported that ITS sequences are genetically constant or show little variation within the species, but vary between species in a genus. The genetic diversity detected

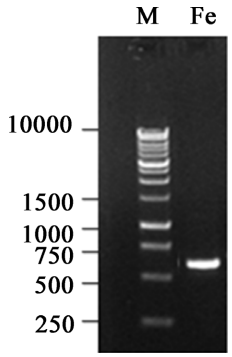


Figure 6. PCR products of the ITS region of *Fusarium equiseti* (*Fe*). M, molecular size marker (1 kb DNA ladder); lane *Fe*, *Fusarium equiseti*.

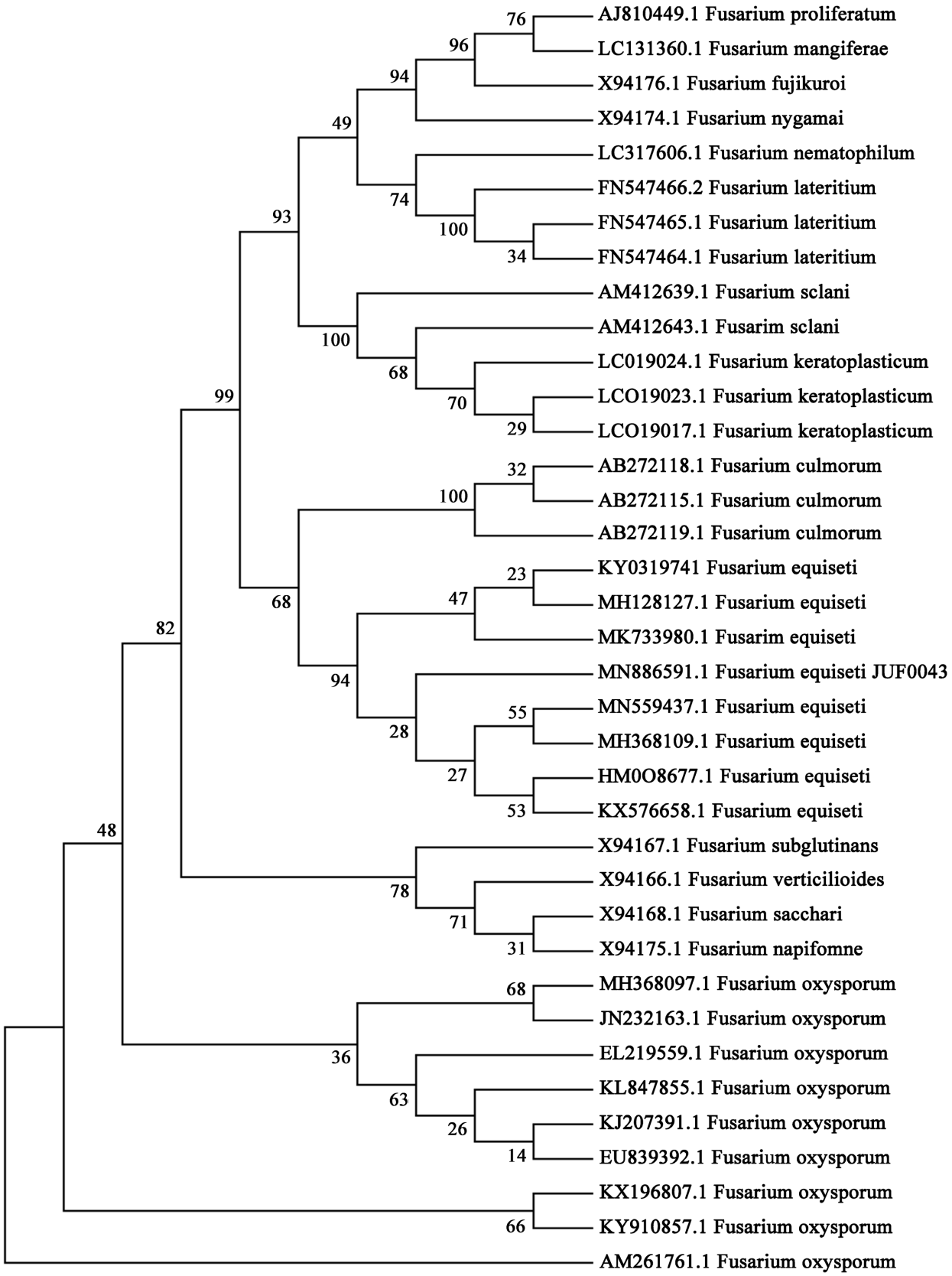


Figure 7. Maximum likelihood tree derived from analysis of ITS sequence dataset of the studied organism with bootstrap value (Bootstrap replication = 1000). Our organism (MN886591.1) marked with "JUF0043".

within groups is probably due to an efficient gene flow and to a high genetic compatibility within the strains tested. Based on the molecular evidence, it is clearly indicated that our studied fungus is *Fusarium equiseti*.

5. Conclusion

Fusarium equiseti was identified as a causal agent of wilt disease in *Centella asiatica* based on classical fungal taxonomy and molecular characterization. PDA was the best culture medium for the mycelial growth and development of *F. equiseti*. Temperature 30˚C was optimum, tested fungi grew well in the neutral condition. The PCR product of the ITS region of *F. equiseti* was 535 bp. Sequence analysis showed that 5.8S of rDNA sequences were identical. The reciprocal homologies of the ITS region sequences ranged from 98% to 100%.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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