

Cultural characteristics and molecular confirmation of *Ustilaginoidea virens* the incitant of false smut of rice

ABSTRACT

Aims: *Ustilaginoidea virens*, which causes false smut of rice (synonyms: green smut or pseudo smut), is an emerging grain disease around the world. The main aim of the study is to find out suitable culturable medium, carbon sources and pH for the growth of false smut pathogen and molecular confirmation of *U. virens* isolates.

Place and duration of study: The *in vitro* studies were conducted in the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, between December 2020 to April 2021.

Methodology: To study the growth pattern of *U. virens*, five different carbon sources and mediums were used. Standardization of pH was done using a PSA medium amended with different pH levels. DNA extraction of all 13 isolates were done using the CTAB method. The phylogenetic tree was constructed using MEGA 12 software

Results: It is a slow-growing pathogen and grew well in the potato sucrose agar medium with a mycelial growth of about (54.00 mm) after 30 days and sucrose was found to be a good carbon source. The pH of 5 was found to be optimum. PCR reaction was carried out to further validate 13 isolates using three sets of primer pairs ITS 1/ITS 4, US1-5/US3-3, and US 2-5/US 4-3 with amplified products of 560 bp, 380 bp and 260 bp. Accession numbers were obtained for all the isolates.

Keywords: Potato sucrose agar medium, pH 5, US 2-5/US 4-3, phylogenetic tree

1. INTRODUCTION

Rice production is frequently subjected to a number of biotic challenges, of which diseases including blast, false smut, sheath blight, stem rot, and bacterial blight are vital to limit the crop's potential output. Worldwide, the average yearly losses from rice diseases are between 15 and 20%. A progressive change in a region's disease pattern occurred as a result of the introduction of high-yielding dwarf varieties and the corresponding change in rice farming methods, including increased plant population, greater fertilizer dosages and more irrigation. Several minor diseases have grown to be of considerable concern. The "False Smut Disease" of rice is one such disease that has attracted widespread notice. The disease is favoured by high relative humidity, low temperatures, rain, and overcast days during flowering [1]. The pathogen *U. virens* targets rice blossoms in particular to produce smut balls [2]. As they develop, mycelia eventually surround the floral organs emerging from the spikelet, and eventually form ball-shaped chlamydospores [3]. Ascospores produced by sclerotia are the initial source of infection for rice plants, although airborne chlamydospores can cause a secondary infection [4]. When a plant is infected, one or more mature grains are replaced with globular, yellowish-green, velvety smut balls [5]. In different parts of India, the disease has grown endemic and has caused losses ranging from 2 to 85 per cent since it first manifested in epidemic

form in 2001 and 2002 on cultivar PR 116 [1,6]. There have been reports of disease incidences from Punjab and Tamil Nadu on various cultivars of roughly 10%–20% and 5-85%, respectively. Its emergence in recent years may be related to high-input farming, greater use of hybrid cultivars and climate change [7]. Hence an attempt was made to standardize the medium for growth of the false smut pathogen and identification at molecular level will leads to proper confirmation of pathogen. This molecular identification will eventually leads to sustainable management of false smut disease.

2. MATERIALS AND METHODS

Pathogen *U. virens* (FS 10) accession number (MZ221826) was used in the entire study.

2.1 Standardization of different solid medium on mycelial growth of *U. virens*

Different solid media, viz. potato dextrose, carrot dextrose, corn meal, oatmeal, and potato sucrose agar, were prepared and used for this study. The pathogen, *U. virens*, was inoculated at the center of the plates by placing an eight mm actively growing culture disc from a 20 day old culture using a sterilized cork borer. The plates were incubated at $(25 \pm 2^{\circ}\text{C})$ for 30 days. Three replications were maintained for each treatment. The radial growth of the mycelium was measured on the 10th, 20th and 30th days after inoculation.

2.2 Growth on different carbon sources

The Czapek's dox agar medium contained various carbon sources, including sucrose, glucose, fructose, sodium carbonate and calcium carbonate, in place of the standard carbon source. The different carbon sources amended media was poured into sterilized Petri plates @ 20 ml and allowed to cool. Using a sterilized cork borer, an eight mm *U. virens* actively growing culture disc was cut from a 20-day-old culture and placed in the center of each Petri plate containing the aforementioned solid media separately, and three replications were maintained. Each Petri plate was then incubated for 30 days at $25 \pm 2^{\circ}\text{C}$. The diameter of mycelium was measured on the 10th, 20th and 30th day.

2.3. Growth at different pH levels

U. virens growth was examined on PSA in different pH ranges. Potato sucrose agar medium was prepared and the pH was adjusted to 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 using 0.1 N HCl or NaOH. The media was then poured into a 250 ml conical flask and sterilized. Under aseptic conditions, the different pH amended medium was poured into the Petri plates and an eight mm *U. virens* actively growing culture disc was placed in the middle of each Petri plate and incubated for 30 days at room temperature ($25 \pm 2^{\circ}\text{C}$). For each pH level, three replications were maintained.

2.4. DNA extraction and molecular confirmation

The total DNA of 13 isolates was extracted using the Cetyltrimethyl ammonium bromide (CTAB) method. Pure culture of all the isolates of *U. virens* mycelia was inoculated in Potato sucrose broth and incubated at 28°C for two weeks. The DNA extraction was done by following the procedure of Ladhakshmi *et al.*, 2012

The ITS region of DNA was amplified using PCR conditions with universal primers reported by [8]. ITS 1 (TCCGTAGGTGAACCTGCGG) and ITS 4 (TCCTCCGCTTATTGATATGC). The cycle parameters included were: for initial denaturation at 96 °C for 2 min, followed by 30 cycles consisting of denaturing at 96 °C for 30 seconds, annealing at 58 °C for 30 seconds and extension at 72 °C for 30 seconds and final extension for 7 min at 72 °C. Specific internal transcribed spacer (ITS) primers (US 2-5/US 4-3) and (US 1-5/, US 3-3) were used to confirm *U. virens* [9]. The polymerase chain reaction (PCR) mixture (20 µl) consisted of 0.5 µl of 2.5 mM of dNTPs, 10 pmol of each primer, one unit of Taq polymerase and DNA template (20 ng). PCR reaction was carried out for false smut-specific primers with an initial denaturation step at 96 °C for 2 min, 30 cycles of amplification (20 s for denaturation at 96 °C, 30 s for primer annealing at 53 °C and 30 s for extension at 70 °C), and one cycle of final extension at 72 °C for 7 min. A Thermocycler was used for the amplification (Eppendorf). Standard agarose gel (1.2 %) electrophoresis was used to examine PCR-amplified products. The gel was documented using a gel documentation device (Bio-rad system), under UV light.

2.5 ITS sequencing and phylogenetic tree

The purified DNA from ITS-PCR was sequenced and subjected to BLAST analysis on <http://www.ncbi.nih.gov/index.html>. The sequences were submitted to the NCBI-Gen Bank database and accession numbers were obtained for all 13 isolates and a phylogenetic tree was constructed using MEGA 12 software.

3. RESULTS AND DISCUSSION

3.1 Growth on different solid medium

In order to assess the suitability of seven different mediums for the mycelial growth of *U. virens*, FS 10 isolate was used to compare the pathogen's growth. The colony diameter was recorded after incubation at 25 °C for 30 days (Table 1). The findings revealed that Potato Sucrose Agar was found to be optimum for fast growth and yielded the maximum growth in diameter of (54.00 mm). The least growth was recorded in oatmeal agar medium (45.66 mm). The results were corroborated with those of [10,11,12,13,14,15], which have a mean mycelial growth of about 56.05 mm, 67.36 mm, 51.6 mm, 69.7 mm, 40 mm and 68.74 mm respectively, in PSA medium after 30 days of incubation. However, [16,17,18,19] reported that potato sucrose agar (PSA) medium was the best medium for the growth of *U. virens*.

3.2 Growth in carbon sources

The presence of carbon in the medium is essential for the sporulation, growth and development of fungi. In the current investigation, different carbon sources utilized showed varying degrees of mycelial growth and the results are presented in (Table 2). The results showed that after 30 days of incubation, sucrose (68 mm) was the optimum carbon source for the mycelial growth of *U. virens* followed by fructose (5.30 cm). The results were in line with [13], who reported that fungal growth was maximum when sucrose was used as a carbon source (56.7 mm). [16] documented the carbon requirements of *U. virens*, indicating that sucrose and starch were the optimum carbon sources. The radial growth rate was 2.6 and 2.2 mm in a day on sucrose and starch, respectively. However, [12] documented that mannitol was the best carbon source.

3.3 Growth at different pH

The pathogen was cultured in a PSB medium at various pH levels to determine the optimum pH for the growth of *U. virens*. Six different pH levels (4.0, 5.0, 6.0, 7.0, 8.0 and 9.0) were tested (Table 3). After 30 days of incubation, the maximum mycelial growth of about (62.5 mm) was recorded at pH 5 followed by pH 6 (51.25 mm). These findings demonstrate that fungus thrives in a pH range of slightly acidic to neutral. The least mycelial growth was observed in pH 8 (31.75 mm). The results were substantiated by [11], who reported that *U. virens* grew well at pH 6 (68.82 mm). [12] reported that the fungus recorded the highest mycelial dry weight at pH 6.5 (560.0 mg) followed by pH 6.0 (360.0 mg). [13 and 14] documented that the highest mycelial dry weight (132.1-171.5 mg) was obtained at pH 6 compared to pH 5.5 and 6.5, [20 and 21] have also documented that pH 6.0 was optimum for mycelial development.

3.4 Molecular confirmation using ITS and specific primers

The molecular level identity of all 13 isolates was confirmed using the primers ITS 1 / ITS 4, US1-5/US3-3, and US 2-5/US 4-3. The identification of the thirteen isolates was validated using PCR confirmation. All the isolates used in the investigation had bands with a specific length of approximately 565 bp, 260 bp and 380 bp, respectively (Fig. 1 and 2). The ITS 1/ ITS 4 amplified product of all the isolates was subjected to partial sequencing, and submitted to NCBI and accession numbers were obtained (Table 4). The NCBI blast result showed that the nucleotide sequence of all thirteen isolates had the identity of a minimum of 90.86 to a maximum of 100 per cent with all *U. virens*. [10,22,23] documented that the false smut pathogen was confirmed using ITS 1 and ITS4 with sequence homology of about 98 to 100 per cent, 91 to 99 per cent respectively. Sixty isolates of *U. virens* showed specific bands approximately at 540 bp for ITS 1 & 4 primers and accession numbers were obtained [24]. The results were corroborated with [13,25] for ITS 1/ ITS 4 with a specific amplification. Similar results for US1-5/US3-3 were documented by [1,9,13,26,27], which also had a specific band for all the *U. virens* isolates at 380 bp. Nevertheless, [1,9,13] have reported that a specific band was seen at 230bp while using US 2-5/US 4-3 specific primer.

3.5 Construction of phylogenetic tree

The phylogenetic tree was constructed using MEGA 12 software based on partial sequencing. In addition, 12 other isolates of *U. virens* sequences were collected from NCBI and true smut *Ustilago tritici* was used as an outgroup (Fig. 3) The phylogenetic position of *U. virens* isolates was compared with different isolates of *U. virens* and true smut fungi which depicted that all the false smut isolates come under one clade and *Ustilago tritici* in a separate clade. The clade was further subdivided into different subclades showing that there was genetic variability among the isolates of *U. virens*.

The phylogenetic tree was constructed using ITS 1 and ITS 4 based on neighbour joining method for 61 *U. virens* isolates, they were grouped into two clusters viz. cluster I consist of 54 isolates and cluster II consists of 7 isolates [24]. [28] constructed phylogeny based on sequences at specific SNP-rich locations and found that there were considerable genetic variations across various regional groups of *U. virens*. [27] reported that using the phylogenetic analysis of ITS sequences, all

the isolates were categorically divided into two groups, with 97–100% sequence similarity, and all *U. virens* were placed into a single cluster. [29] constructed the tree using ITS 1 and ITS 2 sequences and reported that three isolates came under the same clade and one isolate was in a separate clade. [30] have demonstrated that all *U. virens* strains constituted a sub-clade within the Clavicipitaceae clade and clearly distinguished true smut fungi from other *U. virens* strains. [14,27,31] constructed a phylogeny tree based on RAPD primer sequences and confirmed the variability among the isolates.

4. CONCLUSION

The present study concluded that the suitable medium for the mycelial growth of *U. virens* was potato sucrose agar medium under laboratory conditions. The *U. virens* grew luxuriantly in sucrose as the carbon source and at pH 5. The molecular confirmation of all 13 isolates revealed that all were *U. virens*. The phylogenetic tree constructed revealed that the ascomycetes fungus that causes false smut disease of rice, was different from the true smut fungus that belongs to the basidiomycetes family and all the thirteen isolates of *U. virens* have considerable genetic variability.

REFERENCES

1. Ladhakshmi D, Laha GS, Singh R, Karthikeyan A, Mangrauthia SK, Sundaram RM, Thukkaiyannan P, Viraktamath BC. Isolation and characterization of *Ustilagoideavirens* and survey of false smut disease of rice in India. *Phytoparasitica*. 2012; 40(2):171-176.
2. Ashizawa T, Takahashi M, Arai M, Arie T. Rice false smut pathogen, *Ustilagoideavirens*, invades through small gap at the apex of a rice spikelet before heading. *J. of Gen. Plant Pathol.* 2012b; 78:255-259.
3. Tang YX, Jin J, Hu DW, Yong ML, Xu Y, He LP. Elucidation of the infection process of *Ustilagoideavirens* (teleomorph: *Villosiclavavirens*) in rice spikelets. *Plant Pathol.* 2013; 62:1-8.
4. Ashizawa T, Takahashi M, Moriwaki J, Hirayae K. Quantification of the rice false smut pathogen *Ustilagoideavirens* from soil in Japan using real-time PCR. *Eur. J. of Plant Pathol.* 2010a;128(2):221-232.
5. Atia M. Rice false smut (*Ustilagoideavirens*). *Egypt. J. of Plant Dis. and Prot.* 2004; 111(1): 71-82.
6. Pannu PPS, Thind TS, Goswami S. Standardization of technique for artificial creation of false smut of rice and its management. *Indian Phytopath.* 2010; 63: 234-35.
7. Lu D, Yang X, Mao J, Ye H, Wang P, Chen Y, He Z, Chen F. Characterising the pathogenicity diversity of *Ustilagoideavirens* in Hybrid Rice in China. *J. of Plant Pathol.* 2009; 443-451.
8. White TJ, Bruns T, Lee SJ, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications.* 1990; 18(1):315-322.
9. Zhou YL, Izumitsu K, Sonoda R, Nakazaki T, Tanaka E, Tsuda M, Tanaka C. PCR-based specific detection of *Ustilagoideavirens* and *Ephelis japonica*. *J. Phytopathol.* 2003a; 151(9):513-518.
10. Savitha AS, Nagaraja A, Ajithkumar K. Variability studies of *Ustilagoideavirens* isolates causing false smut and its severity in different rice growing ecosystems of Karnataka. *The Pharma Innov. J.* 2022; 11(2): 01-08.
11. Priya B, Amarendrar K, Sanjeev K, Santosh K. Physiological dynamics of *Ustilagoideavirens* causing false smut disease of rice. *Int. J. Curr. Microbiol. App. Sci.* 2018; Spl. Issue-7: 4562-4566.
12. Manasa R. Analysis on specific adaptation of *Ustilagoideavirens* in occupying host florets and their management. Ph. D. Thesis, TN. Agri. Univ., 2018; Tamil Nadu.
13. Pramoda S. Studies on screening germplasm against false smut (*Ustilagoideavirens*) and brown spot (*Bipolarisoryzae*) and their management in rice. Ph. D. Thesis, TN. Agri. Univ., 2016; Tamil Nadu.

14. Baite MS, Sharma RK, Devi TP, Sharma PR, Kamil DE. Morphological and molecular characterization of *Ustilagoideavirens* isolates causing false smut of rice in India. *Indian Phytopathol.* 2014; 67(3):222-227.
15. Rani R, Sharma V K, Lore JS, Pannu PPS. Cultural studies on *Ustilagoideavirens*, the incitant of false smut of rice (*Oryzasativa*). *Indian J. of Agrl. Sci.* 2015; 85 (7):28-31.
16. Fu R, Yin C, Liu Y, Ding L, Zhu J, Zheng A, Li P. The influence of nutrient and environmental factors on mycelium growth and conidium of false smut *Villosiclavirens*. *Afr. J. of Microbiol. Res.* 2013; 7(9):825-833.
17. Xiu-Juan Y, Shu Ting W, Lin G, Xin Y, Chun H, Fu-Ru C. Study on characteristics of the mycelial growth and conidia germination of *Ustilagoideavirens*. *J. of Fujian Agrl. and Forestry Univ.* (Natural Science Edition). 2011; 6:003.
18. Li Y, Li QL, Zheng L, Luo HG, Huang J B, Zhang QD. Characteristics of asexual spore germination and growth of *Ustilagoideavirens* in different media. *Plant pathol.* 2008; 35(1):23-27.
19. Yang L, HangangLQZLL, Qiudong HJZ. Characteristics of asexual spore germination and growth of *Ustilagoideavirens* in different media. *Actaphytophylacasinica.* 2008; 1:6.
20. Jecmen A, 2014. Biology and Control of Rice False Smut Caused by *Ustilagoideavirens* (Teleomorph *Villosiclavirens*) M.Sc. Thesis, Univ. of Arkansas, 2014; Washington.
21. Hashioka Y, Yoshino M, Yamamoto T. Physiology of the rice false smut, *Ustilagoideavirens*(Cke.) Tak. Research Bulletin. Saitama Agricultural Experiment Station. 1951; 2:1-20.
22. Bashyal BM, Parmar P, Zaidi NW, Sunani SK, Prakash G, Aggarwal R. Improved methodology for the isolation of false smut pathogen *Ustilagoideavirens* of rice. *Indian Phytopathol.* 2021; 74(1):249-252.
23. Baite MS, Sharma RK. Isolation technique and culture conditions of false smut pathogen (*Ustilagoideavirens*) on rice. *Indian Phytopathol.* 2015;68(1):50-5.
24. Sharanabasav H, Pramesh D, Prasannakumar MK, Chidanandappa E, Yadav MK, NgangkhamU, Parivallal B, Raghavendra BT, Manjunatha C, Sharma SK, Karthik N. Morpho-molecular and mating-type locus diversity of *Ustilagoideavirens*: an incitant of false smut of rice from Southern parts of India. *Journal of Appl. Microbiol.* 2021; ISSN 1364-5072.
25. Lin X, Bian Y, Mou R, Cao Z, Zhu Z, Chen M. Isolation, identification, and characterization of *Ustilagoideavirens* from rice false smut balls with high ustilotoxin production potential. *J. of Basic microbial.* 2018; 58(8):670-678.
26. Zhou L, Lu S, Shan T, Wang P, Sun W, Chen Z, Wang S. Chemistry and biology of mycotoxins from rice false smut pathogen. In. *Mycotoxins: Properties, Applications and Hazards*; Melborn, BJ, Greene, JC, Eds. 2008b; 109-130.

27. Kumari P, Sharma RK. Histopathological and molecular characterization of *Ustilagoideavirens* in rice. *Indian Phytopathol.* 2015; 70(3): 326-330.
28. Sun X, Kang S, Zhang Y, Tan X, Yu Y, He H, Zhang X, Liu Y, Wang S, Sun W, Cai L, Li S. Genetic diversity and population structure of rice pathogen *Ustilagoideavirens* in China. *PLoS One.* 2013; 8(9):76879.
29. Manu D, Pramoda S, Ramanathan A, Ramchander S, Manonmani S, Jeyaprakash, P, Robin S. Isolation, Characterization and Pathogenesis of *Ustilagoideavirens* Causing False Smut Disease in Rice (*Oryzasativa L.*). *Int. J. Curr. Microbiol. App. Sci.* 2017; 6(7): 632-640.
30. Pramesh D, Prasannakumar MK, Muniraju KM, Mahesh HB, Pushpa HD, Manjunatha C, Saddamhusen A, Chidanandappa E, Yadav MK, Kumara MK, Sharanabasav H. Comparative genomics of rice false smut fungi *Ustilagoideavirens* Jv-Gvt strain from India reveals genetic diversity and phylogenetic divergence. *3 Biotech.* 2020; 10(8):1-14.
31. Bag MK, Ray A, Masurkar P, Devanna BN, Parameswaran C, Baite M, Rath PC, Nayak A. Genetic diversity and population structure analysis of isolates of the rice false smut pathogen *Ustilagoideavirens* in India. *Plant Pathol.* 2021; 70(5): 1085-1097.

Table 1. Effect of different medium on the mycelial growth of *U. virens*

| S.No. | Name of the medium | Radial mycelial growth (mm) | | |
|---------------------|----------------------|---------------------------------|---------------------------------|---------------------------------|
| | | 10 th day | 20 th day | 30 th day |
| 1 | Potato dextrose agar | 24.00 ^{ab} (29.331) | 36.00 ^{ab} (36.862) | 50.00 ^b (45.000) |
| 2 | Carrot agar | 17.00 ^a (24.326) | 25.66 ^c (30.433) | 46.00 ^{bc} (42.705) |
| 3 | Potato sucrose agar | 26.00 ^a (30.646) | 38.00 ^a (38.049) | 54.00 ^a (47.295) |
| 4 | Oatmeal agar | 18.66 ^{ae} (25.596) | 27.33 ^c (31.510) | 45.66 ^c (42.510) |
| 5 | Cornmeal agar | 22.66 ^{bc} (28.422) | 33.33 ^{ab} (35.239) | 47.00 ^{bc} (43.279) |
| 6 | Potato carrot agar | 21.00 ^{cd} (27.271) | 32.00 ^b (34.443) | 46.66 ^{bc} (43.088) |
| CD (P= 0.05) | | 1.819 | 2.847 | 1.944 |

* Values are mean of three replications. Figures in parentheses represent arcsine transformation.

Means in a column followed by same superscript letters are not significantly different according to DMRT at $P \leq 0.05$.

Table 2. Effect of different carbon sources on the mycelial growth of *U. vires*

| S.No. | Carbon sources | Radial mycelial growth (mm) | | |
|---------------------|-------------------|--------------------------------|--------------------------------|--------------------------------|
| | | 10 th day | 20 th day | 30 th day |
| 1 | Sucrose | 27.33 ^a (31.510) | 44.60 ^a (41.930) | 68.00 ^a (55.573) |
| 2 | Fructose | 22.30 ^b (28.179) | 43.00 ^a (40.972) | 62.66 ^a (52.348) |
| 3 | Glucose | 21.00 ^b (27.247) | 35.33 ^b (36.463) | 53.33 ^b (46.913) |
| 4 | Sodium carbonate | 6.00 ^c (14.049) | 12.33 ^c (20.496) | 19.33 ^c (26.055) |
| 5 | Calcium carbonate | 4.66 ^c (13.340) | 6.66 ^d (14.898) | 10.33 ^d (18.720) |
| CD (P= 0.05) | | 3.121 | 3.584 | 3.324 |

* Values are mean of three replications. Figures in parentheses represent arcsine transformation.

Means in a column followed by same superscript letters are not significantly different according to DMRT at $P \leq 0.05$.

Table 3. Effect of different pH on the mycelial growth of *U. vires*

| S.No. | pH levels | Radial mycelial growth (mm) | | |
|---------------------|-----------|---------------------------------|--------------------------------|--------------------------------|
| | | 10 th day | 20 th day | 30 th day |
| 1 | 4 | 14.25 ^{cd} (22.164) | 33.25 ^c (35.213) | 42.25 ^a (40.541) |
| 2 | 5 | 24.25 ^a (29.498) | 45.25 ^a (41.986) | 62.50 ^b (52.242) |
| 3 | 6 | 20.25 ^b (26.742) | 36.50 ^b (37.166) | 51.25 ^b (45.718) |
| 4 | 7 | 19.50 ^b (26.203) | 32.50 ^c (34.756) | 40.75 ^c (39.666) |
| 5 | 8 | 15.50 ^c (23.182) | 24.50 ^d (29.662) | 31.75 ^c (34.291) |
| 6 | 9 | 13.75 ^d (21.744) | 25.50 ^d (30.324) | 35.00 ^d (36.254) |
| CD (P= 0.05) | | 1.141 | 0.973 | 2.143 |

* Values are mean of three replications. Figures in parentheses represent arcsine transformation.

Means in a column followed by same superscript letters are not significantly different according to DMRT at $P \leq 0.05$.

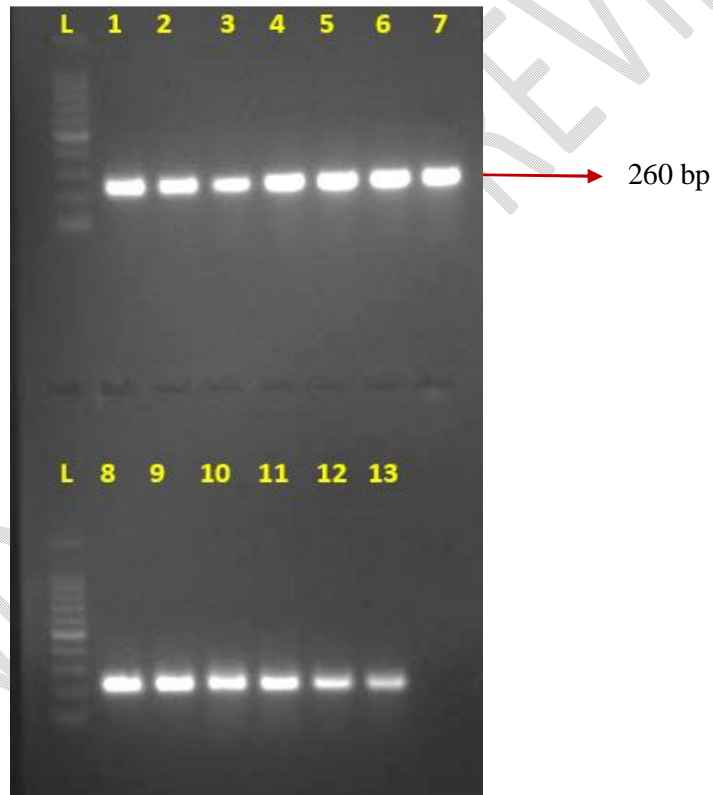
Table 4. Molecular confirmation of *U. virens* using ITS 1 / ITS 4 primers

| Isolates | Acession number | Identified species | Identity (%) |
|----------|-----------------|----------------------------|--------------|
| FS 1 | MZ157262 | <i>Ustilagoidea virens</i> | 99.16 |
| FS 2 | MZ605380 | <i>Ustilagoidea virens</i> | 100 |
| FS 3 | MZ157263 | <i>Ustilagoidea virens</i> | 99.83 |
| FS 4 | MZ133725 | <i>Ustilagoidea virens</i> | 98.39 |
| FS 5 | MZ157280 | <i>Ustilagoidea virens</i> | 99.49 |
| FS 6 | MZ158322 | <i>Ustilagoidea virens</i> | 95.74 |
| FS 7 | MZ646028 | <i>Ustilagoidea virens</i> | 91.79 |
| FS 8 | MZ221827 | <i>Ustilagoidea virens</i> | 91.75 |
| FS 9 | MZ646301 | <i>Ustilagoidea virens</i> | 93.87 |
| FS 10 | MZ221826 | <i>Ustilagoidea virens</i> | 99.24 |
| FS 11 | MZ662821 | <i>Ustilagoidea virens</i> | 100 |
| FS 12 | MZ221831 | <i>Ustilagoidea virens</i> | 100 |
| FS 13 | MZ646351 | <i>Ustilagoidea virens</i> | 90.58 |

→ 380 bp

L- 100 bp DNA marker, 1 – FS1, 2- FS 2, 3- FS 3, 4- FS4, 5- FS 5, 6- FS 6, 7- FS7, 8- FS8, 9- FS9, 10- FS 10, 11- FS 11, 12- FS 12, 13- FS 13

Fig. 1 Polymerase chain reaction for US1-5/US3-3



L- 100 bp DNA marker, 1 – FS1, 2- FS 2, 3- FS 3, 4- FS4, 5- FS 5, 6- FS 6, 7- FS7, 8- FS8, 9- FS9, 10- FS 10, 11- FS 11, 12- FS 12, 13- FS 13

Fig. 2 Polymerase chain reaction for US2-5 / US4-3

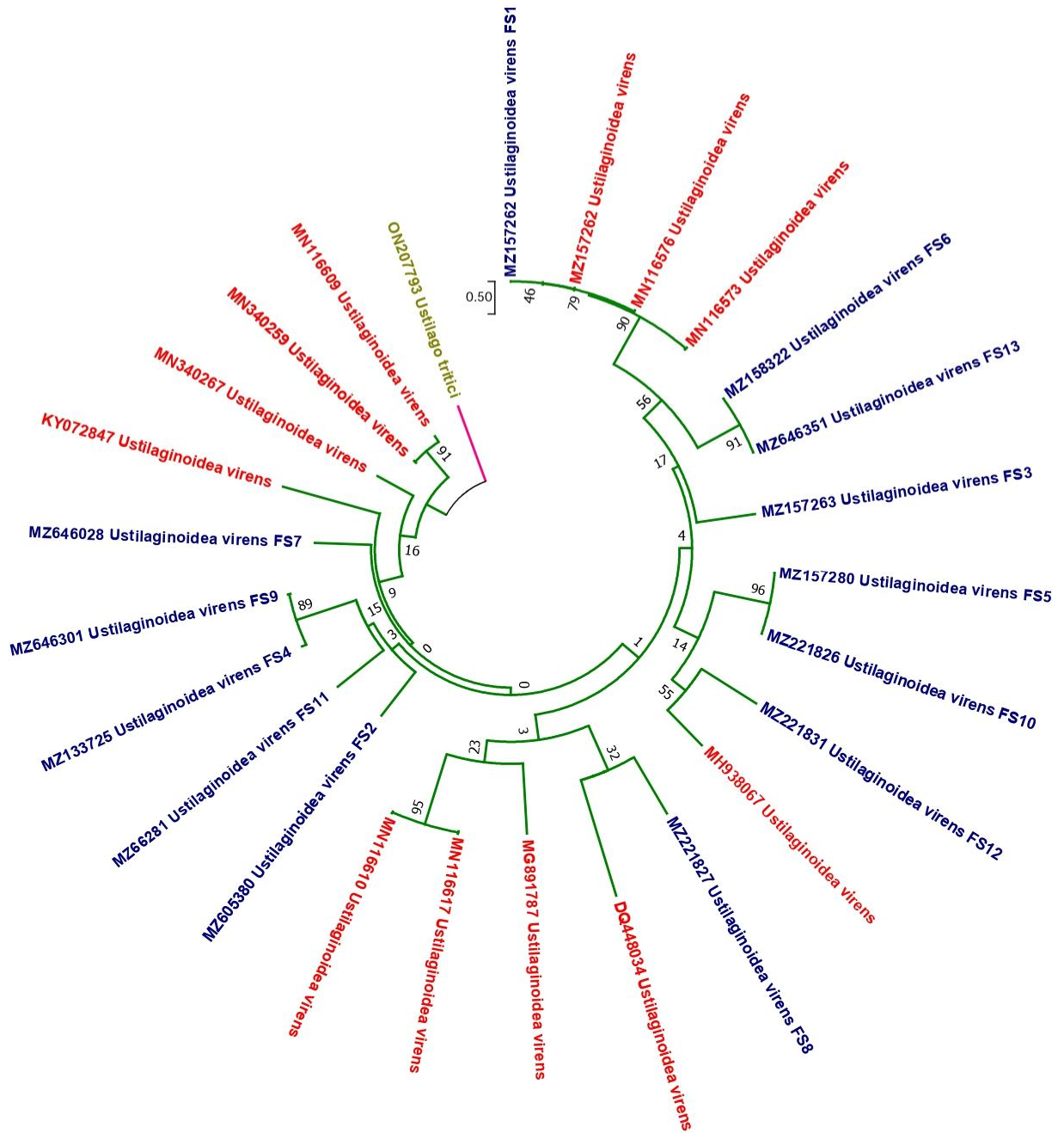


Fig.3 Phylogenetic tree showing genetic variability among different *U. virens* isolates