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Phenotypic and Molecular Identification of Pathogenic *Fusarium* Species Isolated from Various Pulse Growing Geographic Areas of India

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A B S T R A C T

Fusarium phythopathogenic fungi is responsible for high economic loss of cereal food crop. The objective of this study was aimed at isolation, morphological and molecular identification of *Fusarium* species. 13 different *Fusarium* spp. i.e. *F. solani*, *F. chlamydosporum*, *F. tabacinum*, *F. fujikuroi*, *F. oxysporum*, *F. verticillioides*, *F. brachygibbosum*, *Fusarium* sp. and *F. incarnatum* were isolated and identified from diseased samples of chickpea, pigeonpea, rice, lentil and garden pea crop. Colony characteristics like colony color, colony growth diameters, mycelium type, sporulation, pigmentation, odour were obtained after culture purification. Shape, size and septation of microconidia and macroconidia, position, shape, occurrence and size of chlamydospores, conidiophore branching were examined microscopically. MS10, BI01 and KA14 isolates were slow growing, BI02 and UP07 were moderate growing and BI03, HA04, MS06, MS09, MS11 and KA(Gul)13 were fast to very fast growing on PDA after 7-10 days. Chlamydospores were found in most of the isolates. Colonies were abundant, loosely tufted, fluffy, pannose, vinaceous floccose, powdery and some were flat appressed, arachnoid. Pigmentation of most of the isolates was pinkish white to dark pink, carmine to violet in colour. Phylogenetic analysis was done by maximum likelihood method using the ITS-rDNA region of *Fusarium* isolates and multiple sequence alignment of ITS DNA sequences was done using Clustal_W program and all identified sequences were submitted in NCBI GenBank database.

Introduction

Fusarium phytopathogenic fungi is one of the most extensively studied, ubiquitous and destructive phylogenetic diversified soil-borne pathogen around the world which is both saprophytic and pathogenic in nature with a vast host range including horticultural and grain crops that cause diseases like wilt, rot, and damping-off (Martyn, 2014; Zhou *et al.*,

2010; Xiong and Zhan, 2018 and LeBlanc *et al.,* 2017). Members of this species complex are responsible for intense vascular wilt diseases in various plants, and also the cause of contagious diseases in humans and animals, creating a severe challenge to food security and public health (Zhang and Ma, 2017). *Fusarium* species are able to produce one or more mycotoxins which are secondary metabolites with varied amount of toxicity (Bottalico and Perrone, 2002) which can lead to intense immune- suppressive effect due to their multi-inhibitory properties on eukaryotic cells, including suppressing synthesis of DNA and RNA, protein, restrictive of mitochondrial function and disturbing cell division (Rocha *et al.,* 2005). Mycotoxins are also responsible for major storage rots on food and feeds contaminating the substrates (Mohd Zainudin *et al.,* 2008). *Fusarium* wilt is a major threat to production of chickpea since it is seed borne in nature (Jalali and Chand, 1991), it has high survival rate in the soil and on crop residues for up to 6 years as chlamydospores (ICRISAT, 1985) in India. Rice is the second most important cereal food crop grown and exported all over the world (Sunani *et al.,* 2019) and India ranks second in rice production with an area of around 44.6 million hectare (mha) and productivity of 2972 kg/ha (FAO, 2014). Bakanae disease caused by *Fusarium fujikuroi* Nirenberg severely affects quality and production in Basmati rice (Bashyal *et al.,* 2016a, b; Gupta *et al.,* 2015).

The most preferred method is morphological identification of *Fusarium* spp. is microscopic examination under light microscopy where diagnosis depend only upon morphology based on their micro and macroscopic features of diseased tissues. However, these features are mostly reported to be unstable (Booth, 1971).Usually, disease diagnosis is performed first after the appearance of visual symptoms. In disease diagnosis, biochemical and allozyme characteristics etc. are also performed which require high expertise and are still prone to inaccuracy (Kheterpal, 2006). These methods are also time consuming and proved to be limited and insufficient. At present, identification of pathogens is executed based on the nucleotide sequence information from conserved regions using PCR amplification assays, internal transcribed spacer (ITS) region from the conserved ribosomal RNA genes, translation elongation factor (EF-1a), intergenic spacer (IGS), b-tubulin region and the small subunit mitochondrial sequences (mtSSU) have been proved valuable in distinguishing species and origins of *Fusarium* (Baayen *et al.,* 2000; O'Donnell *et al.,* 2000; Skoygaard *et al.,* 2001). This sequence information has been extensively used in the taxonomy and phylogenetic study of *Fusarium* species by identifying and discriminating isolates within a species. These molecular techniques based on DNA have been extensively used and has proved to be more rapid, sensitive and precise detection method (Sunani *et al.,* 2019).

Therefore, the objectives of this present study were to isolate and identify disease causing *Fusarium* species from infected parts of crop plants based on the morphological data and molecularly using internal transcribed spacer (ITS).

Materials and Methods

The infected stems, seeds and roots were congregate from chickpea, pigeon pea, rice, lentil and garden pea fields. They were collected from different states of India i.e. Bihar, Maharashtra, Karnataka, Uttar Pradesh, Haryana and Himachal Pradesh.

Isolation of *Fusarium* spp.

5 to 10 cm segments of infected stems were collected and brought to the laboratory for isolation. Diseased stems, seeds and roots

were aseptically cut into 1-cm pieces and were surface sterilized in 10% (w/v) sodium hypochlorite solution for 1 min each. They were rinsed in sterile distilled water and blotted dry in sterilized blotting paper then placed onto potato dextrose agar (Himedia) plates by tissue segment method (Rangaswami, 1958). PDA media was amended with streptomycin sulfate to remove bacterial contamination. Plates then were at 25°C in the dark. The *Fusarium* cultures were purified by repeated subculturing on PDA (Leslie and Summerell, 2006) and pure cultures were maintained at 4°C in PDA slants.

Morphological identification of *Fusarium* isolates

Morphological identifications of isolates were made using the criteria of Gerlach and Nirenberg (1982) and Leslie and Summerell (2006). Culture characteristics were determined by using Methuen handbook of colour chart (Kornerup and Wancher, 1978). Shape, size and septation of microconidia; shape, presence or absence of microconidia and presence or absence of chlamydospores based on Summeral *et al.,* (2003) were taken.

Molecular identification of *Fusarium* isolates

Isolation of DNA

Genomic DNA was extracted by method described by Raeder and Broda (1985). For DNA extraction, 4 days old culture of *Fusarium* isolates were scraped from PDA plates and transferred into centrifuge tubes carrying 25ml pre heated (65^{o}C) lysis buffer. The tubes were incubated at 65^{o}C for an hour with occasional stirring by vortexing every 15 min followed by addition of equal volume of Phenol: Chloroform: Iso-amyl alcohol (25:24:1). Samples were centrifuged at 15,000 rpm for 10 minutes at room temperature. Upper aqueous phase was precipitated with 2 volumes of ice-cold ethanol and 0.1 volume of 3M sodium acetate (pH 5.20) and incubate for 1 hr at 4^{o}C and spinned at 15,000 rpm for 15 minutes at room temperature and the pellets obtained were washed with 70% ethanol and again spinned at 10,000 rpm for 10 minutes. Nucleic acid was eluted by elution buffer (10mM TrisHCl and 1mM sodium EDTA, pH 8). The DNA was dissolved and it was treated with 10 µl RNase and incubated for 1 hr at 37^{o}C, after incubation 500 µl of water saturated phenol: chloroform: isoamyl alcohol (25: 24: 1) was added and centrifuged at 10,000 rpm for 20 min.

The upper aqueous phase was collected and 50 µl of 3M sodium acetate and 1 ml ethanol was added. Isolated DNA was incubated overnight or at -80°C for 1 hr. Supernatant was discarded and the pellet is washed with 70% ethanol, air dried and approx. 100 µl of 1X TE buffer was added to dissolve the pellet. DNA was then stored at -20°C for PCR.

Molecular characterization using ITS

Molecular identification of *Fusarium* cultures were carried out by amplification of internal transcribed spacer 1, partial sequence, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence and 28S ribosomal RNA gene, partial sequence using primer pair ITS1 (5’ TCCGTAGGTGA ACCTGCGG-3’) and ITS4 (5’-TCCTCCG CTTATTGATATGC-3’) (White *et al.,* 1990).

The ITS region was amplified with an initial denaturation for 5 minutes at 95°C, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing of primers at 60°C for 30 seconds, and extension at 72°C for 30 seconds, and the amplification was completed with one cycle of final extension at 72°C for 5 minutes in PCR thermocycler (Bioer) using 25

µl PCR mastermix containing 60–100 ng genomic DNA, 2.5 µl of 10X buffer (10 mMTris–HCl, pH 8.8), 2 mM each of dNTP, 2.5 mM MgCl2, 25 pmol ml^{-1} primer (each primer) and 1U of Taq DNA Polymerase. Amplified products were visualized on agarose gel (1.5%) in 1X TAE buffer. The gel image was taken using gel documentation system.

Sequencing and Phylogenetic analysis

Unpurified PCR product was sent to Xcelris Labs, Gujarat, India for sequencing. The partial ITS sequence thus obtained was analyzed using BLASTn (Basic Local Alignment Search Tool for Nucleotide Sequences) with NCBI (National Center for Biotechnology information; www.ncbi.nih.gov), GenBank database to assess its identity. Alignment of ITS DNA sequences was done using Clustal_W program (Vincelli and Tisserat, 2008). The evolutionary analysis was conducted in MEGA X (Kumar *et al.,* 2018) and was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993). This analysis involved 13 nucleotide sequences. There were a total of 524 positions in the final dataset.

Results and Discussion

Isolation and morphological identification of *Fusarium* isolates

13 *Fusarium* spp. were isolated from diseased crop plants. The details of location, host of *Fusarium* isolates are shown in table 1.

Based on colony characteristics and microscopic structures of microconidia, macroconidia and other morphological characters, BI01 was identified as *F. solani*, BI02 as *F. chlamydosporum*, BI03 as *F. fujikuroi*, HA04 as *F. fujikuroi*, MS05 as *F. tabacinum*, MS06 as *F. solani*, UP07 as *F. oxysporum*, MS09 as *F. verticillioides*, MS10 as *F. brachygibbosum*, MS11 as *F. solani*, KA(Gul)13 as *Fusarium* sp., KA14 as *F. solani* and HP(Ham)15 as *F. incarnatum*(Table 2).

Molecular identification based on ITS

PCR amplification of ITS region generated bands ranging from 460-504 bp *Fusarium* sequences thus obtained were compared with sequences from NCBI database using BLASTn.

These sequences thus identified were deposited in NCBI GenBank and accession numbers were allotted. Table 1 shows isolate code with GenBank accession numbers.

Isolate BI01, MS06, MS11 and KA14 were identified as *F. solani* (KP881505, KP881510, KP881514 and KP881516 respectively), BI02 as *F. chlamydosporum* (KP881506), BI03 and HA04 as *F. fujikuroi* (KP881507 and KP881508), MS05 as *F. proliferatum* (KP881509), UP07 as *F. oxysporum* (KP881511), MS09 as *F.verticillioides* (KP881512), MS10 as *F. brachygibbosum* (KP881513) and KA(Gul)13 and HP(Ham)15 as *Fusarium* sp. (KP881515 and KP881517).

The evolutionary history of *Fusarium* isolates was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993).

The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 13 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 524 nucleotide sites in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al.,* 2018).

Table.1 Location, host, morphological and sequenced based identified species with GenBank accessions of *Fusarium* isolates

Isolate code	Location	Host plant	Morphological identification	Sequence based identification	GenBank Accession No.
BI01	Samastipur, Bihar	Lentil stem	*Fusarium solani*	*Fusarium solani*	KP881505
BI02	Samastipur, Bihar	Lentil stem	*Fusarium chlamydosporum*	*Fusarium chlamydosporum*	KP881506
BI03	Samastipur, Bihar	Lentil stem	*Fusarium fujikuroi*	*Fusarium fujikuroi*	KP881507
HA04	Kaithal, Haryana	Rice stem	*Fusariumfujikuroi*	*Fusariumfujikuroi*	KP881508
MS05	Badnapur, Maharashtra	Pigeon pea stem	*Fusarium tabacinum*	*Fusariumproliferatum*	KP881509
MS06	Bori, Maharashtra	Chickpea stem	*Fusarium solani*	*Fusarium solani*	KP881510
UP07	Kanpur (U.P.)	Chickpea seed	*Fusarium oxysporum*	*Fusarium oxysporum*	KP881511
MS09	Badnapur, Maharashtra	Chickpea stem	*Fusarium verticillioides*	*Fusarium verticillioides*	KP881512
MS10	Jalna, Maharashtra	Pigeon pea stem	*Fusarium brachygibbosum*	*Fusarium brachygibbosum*	KP881513
MS11	Jalna, Maharashtra	Pigeon pea stem	*Fusarium solani*	*Fusarium solani*	KP881514
KA(Gul)13	Gulbarga, Karnataka	Pigeon pea stem	*Fusarium sp.*	*Fusarium* sp.	KP881515
KA14	Raichur, Karnataka	Pigeon pea root	*Fusarium solani*	*Fusarium solani*	KP881516
HP(Ham)15	Hamirpur (H.P.)	Garden pea stem	*Fusarium incarnatum*	*Fusarium* sp.	KP881517

Fig.1 Phylogenetic tree generated by Maximum Likelihood method using the ITS region nucleotide sequence of the conserved ribosomal DNA of *Fusarium* isolates.

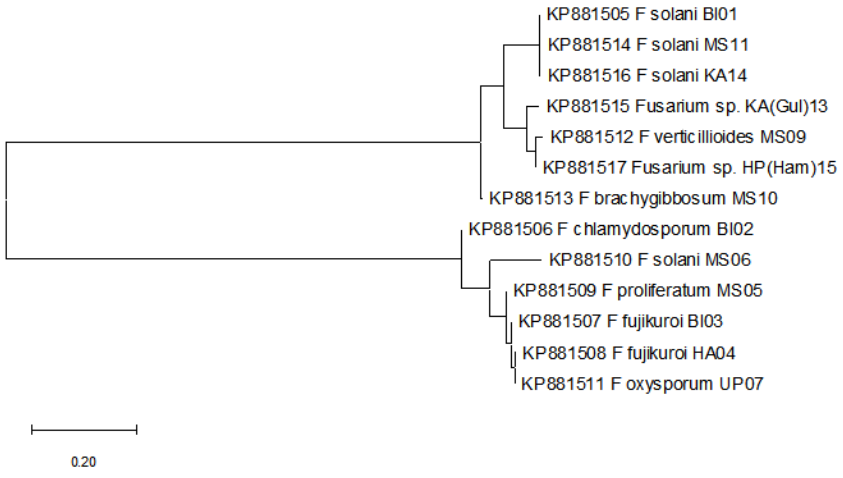


Table.2 Anamorphic characteristics of *Fusarium* isolates

Isolate code	Colonieson PDA	Mycelium type	Pigmentation	Sporulation	Conidiophore	Conidia	Chlamydospore
BI01	slow growing, reaching 4.0-4.2 cm diam. in 10 days	floccose or tufted, cottony	whitish to light pink	Less	Formed in the aerial mycelium, first unbranched, later loosely long branched sometimes densely aggregated	almost straight to slightly curved, thick walled, widest at centre, cuneiform, 0-2 septate (3.5-5.2µm)	round walled, globose to subglobose, single (5-12µm), terminal
BI02	moderate growing, reaching 5-5.2 cm diam. in 7 days	profuse, densely floccose, powdery at surface	pink, carmine	microconidia formed after 4 days	primary conidiophore arising at right angle from main conidiophore	microconidia ovoid, 1-3 septate (12-15 x 3.0-3.2µm), widest at centre	Many, globose to subglobse, terminal or intercalary seen, single (7-17µm) or in pair and in chain
BI03	fast growing, reaching 7.5-8 cm diam. in 7 days	loosely pannose, vinaceous	dark violet	microconidia start forming quickly and later macroconidiaformed	primary conidiophores arising laterally from hyphae in the aerial mycelium	1 or 2 septate(4-13 x 1.5-4.5 µm) microconidia, macroconidia slender, falcate or straight	Absent
HA04	very fast growing, reaching 8-9 cm diam. in 7 days	pale vinaceous	dark pink to violet	abundant macroconidiaand microconidia formed	primary conidiophores arising from hyphae, secondary conidiophores are densely branched	straight microconidia 1 or 2 septate (4-13 x 1.5-4.5 µm), sickle shaped macroconidia, 3-5 septate (19-52 x 2.0-4.2 µm),	Absent
MS05	moderately fast growing, reaching 7-8 cm diam. in 7 days	fluffy, arachnoid, floccose	pinkish white	abundant	at first arising in the aerial mycelium as lateral phialides, sparsely branched	mostly single septate (5-16 x 2.1-4.1µm), slightly curved, with pointed apical cell, 1 celled with 2 vacuoles, variable shape	Not formed

MS06	fast growing, reaching 7.8-8.2 cm diam. in 10 days	floccose, sparse, cottony, creamish white	Dull white	Abundant macroconidia	primary conidiophores unbranched, secondary conidiophore densely	falvatemacroconidia, 3-5 septate (20-38 x 3.3-6.0 µm) microconidiaoval, ellipsoid, reniform, 0-1 septate (6-10 x 2.7-3.3µm)	Mostly seen, terminal or intercalary, globose to subglobose
UP07	moderate growing, reaching 6.5-7.0 cm diam. in 8 days	dense, arachnoid, pink peach with violet tinge	rose pink to strong purple	microconidia emerging from aerial mycelium in false heads, macroconidia solitarily scattered	primary conidiophores short, secondary conidiophore dense, verticillately branched	microcondia 1 or 2 septate (8-22 x 2.3-4.5 µm), ellipsoid, oval, slightly curved, macroconidia falcate, subcylindric, mostly 3septate (18-54 x 2.7-6.0 µm),	abundant, in hyphae and conidia, terminal seen, rough walled, globose to subglobose, single.
MS09	very fast growing, reaching 7.8-8.2 cm diam. in 8 days	abundant, loosely tufted, white pinkish later vinaceous	dark vinaceous, pink to purple	starts quickly, microconidia in long chains, bearing macroconidia lately	primary conidiophores arising from hyphae of aerial mycelium, secondary conidiophores densely branched	microconidia sharp pointed at corners, 1 or 2 septate (4-19 x 1.5-4.6 µm), macroconidia, broadest just below the apical cell, constricted, 3 septate (18-56 x 2.1-4.0 µm)	Absent
MS10	slow growing, reaching 5.4-6.5 cm diam. in 8 days	powdery, white and rose, concentric ring, violet at centre	pink to dark	late conidia formed	microconidia lacking in aerial mycelium	microconidia 1-2 septate mostly (1.8-3.6 x 11.1-15.8 µm), ovoid to fusiform, septate spores slightly curved, scattered macroconidia not very abundant, 2 septate seen,	terminal or intercalary, rough walled, single seen, globose, generally 1-celled (10-15.1 µm), granular
MS11	fast growing, reaching 7.8-	floccose, cottony, felt	pink, later red-brown	starts very quickly in aerial mycelium,	primary conidiophores arising laterally from	microconidia oval, ellipsoid, 0-1 septate	globose to subglobose,

	8.2 cm diam. in 8 days	like, white to pinkish		microconidia cohering in false heads, macroconidia formed profusely	hyphae of aerial mycelium, secondary conidiophores densely, irregularly branched	(µm), macroconidia straight cylindrical central part, with elongated, hooked, pointed corners 3-5 septate (µm)	single (6-10µm) seen, smooth walled, attached to hyphae
KA(Gul)13	fast growing, reaching 7.5-8.0 cm diam. in 10 days	densely floccose to funiculose, arachnoid	pinkish, become rosy to purple	starts quickly, confluent growth	initially arising as single lateral phialide, later in pionnates densely verticillately branched,	thin-walled ovoid, fusoid, 1 or 2 celled conidia, taller macroconidia 3 septate (17-42 x 2.0-3.9 µm),	globose to subglobose, smooth walled, single (8-12 µm) or in clusters
KA14	slow growing, reaching 5.5-6.0 cm diam. in 7 days	floccose, felt-like, pink to light greyish colony	red- brown, greyish	starts quickly in aerial mycelium, abundant macroconidia early formed	primary conidiophores arising laterally from hyphae of aerial mycelium, secondary conidiophores densely, irregularly	microconidia oblong, oval, single or no septate (8-30 x 3.0-4.2 µm), subcylindric, macroconidia falcate, 2-3 septate (20-43 x 3.3-6.0 µm), pointed apical cell or curved at corners	terminal seen, rough-walled, globose to subglobose, single (5-9 µm).
HP(Ham)15	fast growing, reaching 7.5-8.0 cm diam. in 10 days	abundant, densely floccose, powdery, pinkish white colony	whitish, peach	scattered only in aerial mycelium	loosely, irregularly branched, phialidesmonophialidic	thick walled, hook shaped apical cell at corners, mostly 3 septate (13-43 x 2.5-6.5 µm) macroconidia and 2 septate (8-22 x 2.0-4.5 µm) microconidia	terminal seen, rough-walled, single (5-8 µm), globose to subglobose

Fig.2 Multiple sequence alignment of 13 different *Fusarium* sp. isolates using conserved ribosomal ITS region. Insertion/deletion are indicated by dashes (-) and identical nucleotide by dots (.)

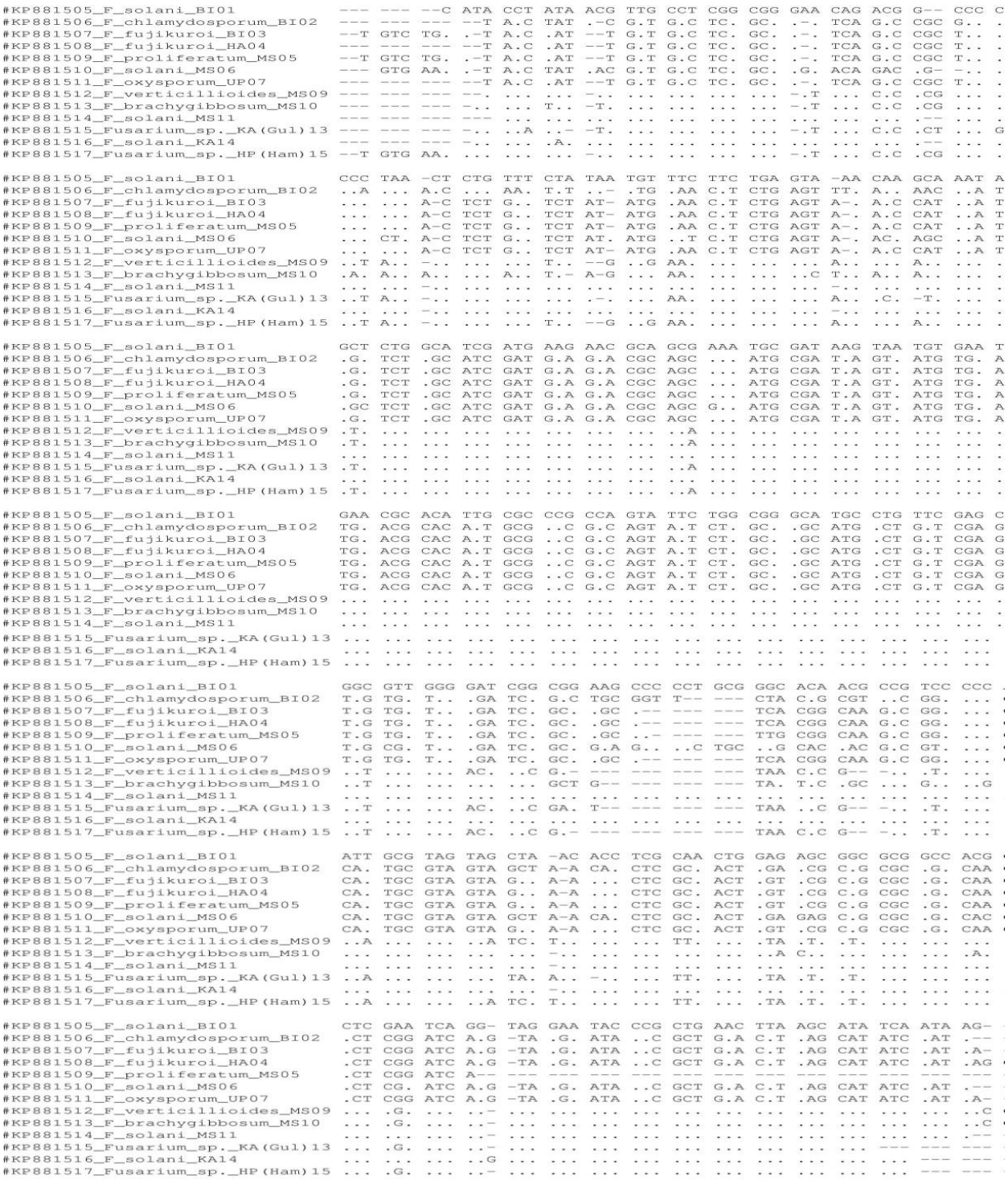
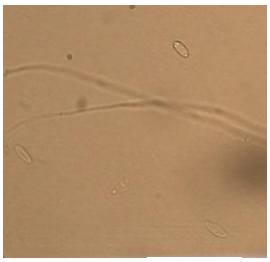
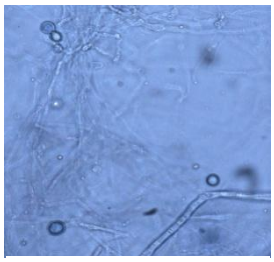


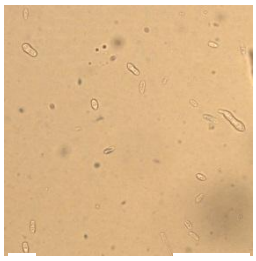
Fig.3 Typical microscopic photos of microconidia, macroconidia and chlamydospores of *Fusarium* isolates, scale bar length-20µm



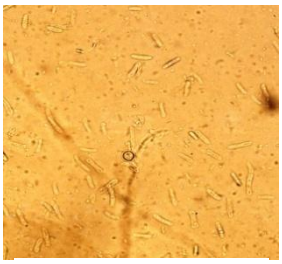
***F. solani* (BI01)**



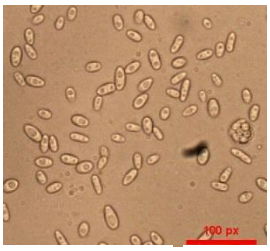
*F. **chlamydosporum*** **(BI02)**



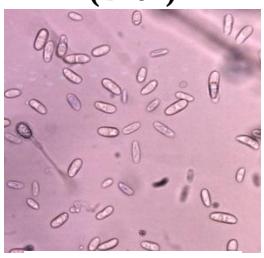
F. fujikuroi **(BI03)**



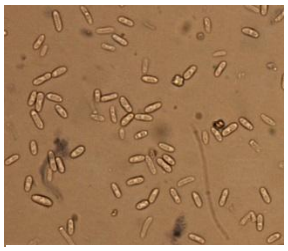
*F. **fujikuroi*****(HA04)**



*F. **tabacinum*****(MS05)**

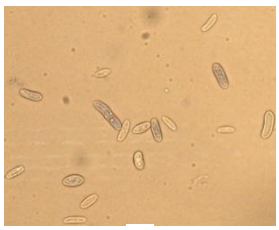


*F. **solani*****(MS06)**

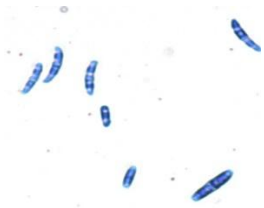


*F. **oxysporum*****(UP07)**

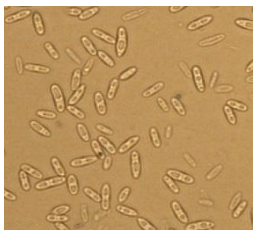
*F. **verticillioides*** **(MS09)**



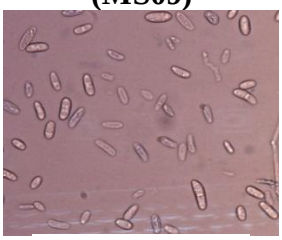
*F. **brachygibbosum*****(MS10)**



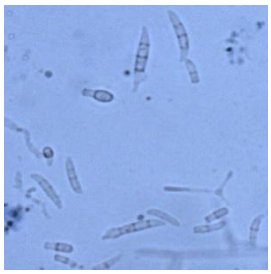
*F. **solani*****(MS11)**



Fusarium **sp.(KA(Gul)13)**



*F. **solani*****(KA14)**



*F. **incarnatum*** **(HP(Ham)15)**

Phylogenetic analysis of the strains based on maximum likelihood method resulted into two major clusters which are further divided into two subclusters each. Subcluster I of Cluster I formed with three *F. solani* and subcluster II of cluster I formed with two *Fusarium* sp. and one *F. verticillioides.* Subcluster I of cluster II formed with one *F. solani* and subcluster II of cluster II formed with one *F. proliferatum*, two *F. fujikuroi* and one *F. oxysporum*. *F. brachygibbosum* and *F. chlamydosporum* were separated from both clusters (Figure 1). ITS sequences of all *Fusarium* isolates were aligned with the consensus region using CLUSTAL W program. The sequences are padded with gaps (dashes) so that wherever possible, columns contain identical characters from the sequences involved. Local alignment

methods find related regions within sequences, they can consist of a subset of the characters within each sequence.

Sequence alignment is used to study the evolution of the sequences from a common ancestor such as protein sequences or DNA sequences. Mismatches in the alignment correspond to mutations, and gaps correspond to insertions or deletions (Figure 2).

In the present study, all 13 isolates of *Fusarium* spp. were morphologically identified and species determination was confirmed by PCR with primers specific for the ITS‑rDNA region. Nucleotide sequences were later deposited with the NCBI GenBank database. The results showed that, out of the 13 isolates, four were *F. solani*, two were F. fujikuroi, two were *Fusarium* sp., remaining each of one isolate was *F. chlamydosporum, F. proliferatum, F. verticillioides, F. oxysporum and F. brachygibbosum* are challenging to identify and to establish systems of taxonomy, because of strains were morphologically similar.

All *Fusarium* sp. were plant pathogens (Chandra *et al.,* 2009). Isolate MS05 was identified as *F. tabacinum* morphologically but it was identified as *F. proliferatum* molecularly. To overcome these limitations of classical taxonomic and morphological characters for species discrimination of the genus *Fusarium,* molecular approaches to differentiate taxa is emphasized. Molecular tools like ITS provide necessary information required for a taxonomic purpose for species identification, as well as to elucidate the evolutionary relationships among species (Singha *et al.,* 2016) and are reliable (Nadia *et al.,* 2021).

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