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Morpho-molecular and Pathogenic Variability of Wilt of Lentil from Indo-Gangetic Plains of India

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ABSTRACT

Background: Fusarium wilt in lentil (*Lens culinaris* L.) infected by *Fusarium oxysporum* is considered as one of the major biotic factors for low productivity in lentil. The present study was conducted to determine nine Fusarium isolates (W1-W8 and W10) which were isolated from different lentil growing fields of West Bengal, India and were characterized for their physio-morphological, biochemical and molecular traits *in vitro*.

Methods: Nine *Fusarium* infected lentil plant samples were collected from 9 different places of West Bengal. Pathogen was isolated from lentil samples using PDA media followed by cultural, morphological, pathogenic and molecular variability studies for all the isolates of *Fusarium.* Type of pigmentation, sporulation, conidia size of each isolate was recorded by observing culture plate after complete growth of the mycelium. Pathogenicity test were done to check the aggressiveness of all the 9 isolates of *Fusarium* in three varieties of lentil (Maitri, HUL-57 and BM-6). The isolated fungal DNA was amplified with specific primer pair, Forward primer ITS 1 and Reverse primer ITS 4.

Result: Morphological assessment of 9 isolates showed distinct identification characters and variability. The mycelia of all the isolates were septate, profusely branched with differential pigmentation, texture and margin.All the Fusarium isolates produced micro-and macro-conidia in pure culture within seven days after inoculation and showed different morphological characters in pathogenicity test, variety Maitri showed better germination (100% in W2, W4, W6, W7, W8 and W10) and less disease severity against most of the strains (75% in W1) as compared to other two varieties against and hence variety Maitri taken into consideration for greenhouse and field trials. Furthermore, identification of molecular variability was performed by sequence analysis of rDNA-ITS region of each isolate and sequencing of rDNA-ITS region supported the morphological study and confirmed the associated fungi at species level.

Key words: Fungal morphology, *Fusarium oxysporum*, Lentil, Pathogenicity.

INTRODUCTION

Lentil (*Lens culinaris* (L.), 2n=2x=14) belonging to the family Fabaceae is an important legume food crop which is valued for its beneficial effects in improving soil fertility (Das *et al*., 2017). The seeds are rich in protein (30%), vitamins and minerals and also good source of nutrients like phosphorus, calcium and iron (Pal *et al*., 2016). The yield losses of lentil are mainly by insects and diseases range from 5 to 10% in temperate regions and 50 to 100% in tropical regions (Hiremani and Dubey, 2016; Mondal *et al*, 2021). Among the diseases affecting lentil, wilt caused by *Fusarium oxysporum* considered as one of the most devastating diseases which is responsible for its low productivity (Tosi and Cappelli, 2001, Sarwar *et al*., 2014). In India this disease may cause 5-10% yield losses but sometimes severe damage may result complete crop failure under favorable conditions for disease development (Chaudhary and Amarjit, 2002; Majumder *et al*., 2022). The major symptoms of the disease are curling of the leaves starting from the lower end and extending upwards, drooping and eventually death of the plant. The present study aimed at characterization of the *F. oxysporum* isolates collected from different lentil growing parts of West Bengal using morphological characters and aggressiveness.

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MATERIALS AND METHODS

Fungal cultures

Nine isolates of *Fusarium oxysporum* addressing nine lentil growing districts of West Bengal, India was collected, isolated by using PDA in BCKV, Pathology Laboratory, Division of Plant Pathology for the year of 2019-20. The fungal cultures were purified and single-spore cultures were maintained at 4°C on potato dextrose agar (PDA) medium and sub-cultured at periodical intervals.

Morphological variability

All 9 isolates of *Fusarium oxysporum* collected from different lentil growing districts of West Bengal, India (Das *et al*., 2022) was studied for their morphological variability. To study cultural characteristics, 5 mm mycelia bits of each isolate were taken from the actively growing cultures and centrally placed on 90 mm Petri plates containing PDA medium. For colony characters like colony diameter, colour, texture and pigmentation were studied and for spore dimensions length and width of both micro conidia and macro conidia and septation in macro conidia were recorded by following Ocular micrometry.

Aggressiveness

The pathogenicity of *Fusarium* isolates was done separately for their aggressiveness by following standard soil infestation method (Rubayet *et al*., 2017) in pot culture under the shade condition on three susceptible lentil cultivars *viz*. Maitri, HUL-57 and BM-6. The experiments were carried out in a greenhouse under controlled conditions (25°C and >80% humidity with continuous light). Ten seeds were sown in plastic pots (15 cm diameter) containing mixture of sterilized soil and vermicompost. The culture of each Fusarium isolate was prepared by inoculating them into maize kernels, then incubated at 25±2°C for 10 days, after which periodic observations were taken for the percentage of disease incidence for each isolate.

DNA isolation

50 mg mycelial mat from 7 days old PDB culture was collected by straining in filter paper and crushed with liquid nitrogen then 600 µl Buffer FG1 added to this powdered dried tissue vortex vigorously to mix. Incubated at 65 p C for 30 minute and aspirate 300 µl supernatant to a new 1.5 ml microfuge tube. Adding 150 µl Buffer FG 2 followed by 300 µl absolute ethanol and vortex. Add 400 µl of Buffer BL into the spin column, incubated at room temperature for 2 minutes, centrifuged at 12000 rpm for 2 minutes and discard the flow through. Transfer column to a second collection tube and wash by adding 650 µl DNA Wash Buffer diluted with absolute (96%-100%) ethanol. Centrifuge at 10,000 g for 1 minand discard the flow-through liquid. Transferred column to a clean 1.5 ml microfuge tube and 100 µl Elution Buffer added and the centrifuge tube for further use. DNA concentration was estimated using an UV vis spectrophotometer (Thermo Spectronic UV1). It was then stored at -20°C until further use (Datta *et al*., 2011).

Data analysis

The data were analyzed statistically by following appropriate designs for each of the experiments. The wilt incidence in the pathogenicity test was analyzed by CRD test (Tukey, 1949) and the ranking was also given for each isolate.

RESULTS AND DISCUSSION

Morphological variability

Single spore-cultures of 9*Fusarium*isolates which were collected from different locations (Fig 1) showed cultural variability in respect of morphology of mycelium, colony colour, texture and margin on PDA. Colour of the colony showed differences among the isolates on PDA media (Fig 2). Colony colour varied from white or pink-white and with time they changed to light brown colour with increased in the age of the fungal cultures within the media (Table 1). Colony diameter was found highest in case of Fusarium isolate W2 (8.87 cm) and minimum colony diameter was noticed in the isolate W3 (8.28 cm). Nine different isolates showed their variability in terms of their conidia size (Fig 3 and Table 2). The maximum average length of micro conidia was found in W2 (15.07±0.60 µm) and minimum length of micro conidia was noticed in W8 (12.69±1.03 µm. In case of breadth of micro conidia, the maximum breadth was noticed on W6 (3.52±0.35 µm) and minimum in W1 (2.85±0.07 µm) and

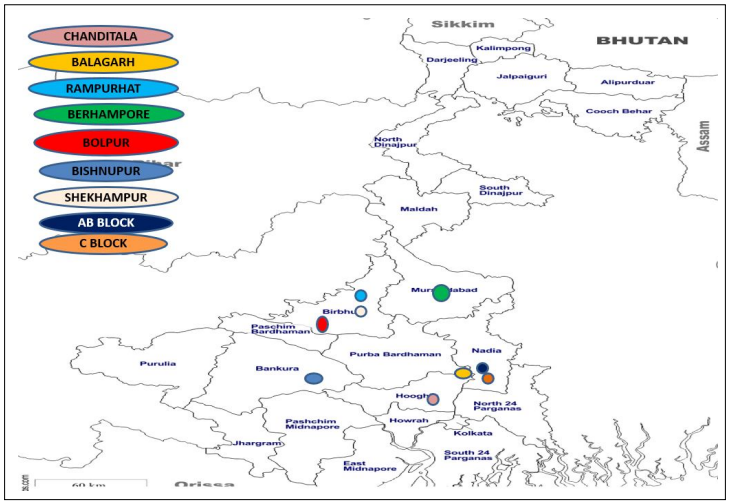


Fig 1: The map of areas where *Fusarium oxysporum* isolates were collected from Lentil growing areas of West Bengal State, India.

Table 1: Cultural studies of *Fusarium* sp. on PDA media.

Isolates	Location	NCBI accession number	Shape	Margin	Texture	Mycelia colour	Full plate growth in days	Conidia shape	Chlamydospore	Sporulation	Colony diameter (cm)
W1	Chanditala	MN758598	Regular	Regular Wavy	Fluffy	Pinkish White	7 days	Macro and Micro	-	++++	8.44
W2	Balagarh	MN758599	Regular	Regular Entire	Velvety	Cottony White	5 days	Macro and Micro	+	+++	8.87
W3	Rampurhat	MN758600	Irregular	Regular Entire	Velvety	Orange White	6 days	Macro and Micro	-	++	8.28
W4	Bolpur	MN758601	Regular	Regular Entire	Fluffy	Creamy White	7 days	Macro and Micro	+	+	8.73
W5	Shekhampur	MN758602	Regular	Regular Entire	Velvety	Purplish White	7 days	Macro and Micro	-	++	8.47
W6	Bishnupur	MN758603	Regular	Regular	Flat	Purplish White	6 days	Macro and Micro	+	++	8.46
W7	Berhampore	MN758604	Regular	Regular Entire	Fluffy	Creamy White	7 days	Macro and Micro	-	++++	8.76
W8	AB Block	MN758605	Regular	Iregular Entire	Sparse Velvety	Cottony White	7 days	Macro and Micro	-	+++	8.61
W10	C Block	MN818591	Irregular	Regular	Fluffy	Purplish White	7 days	Macro and Micro	-	+++	8.80
											SE(m)± 0.25
											CD at 5%NS

++++ Excellent (20 conidia per microscopic field).

+++ Good (15-20 conidia per microscopic field).

++ Fair (10-15 conidia per microscopic field).

+ Poor (10 conidia per microscopic field).

+ Chlamydospore present, -Chlamydospore absent.

Table 2: Variability in conidial shape and size of *Fusarium* sp.isolates grown on PDA.

Isolates	Micro conidia			Macro conidia		
	Length (µm)	Breadth (µm)	Septa (nos.)	Length (µm)	Breadth (µm)	Septa (nos.)
W1	12.77±0.65	2.85±0.07	0-2	21.57±0.528	5.76±0.21	0-3
W2	15.07±0.60	2.97±0.28	0-1	29.65±0.63	3.93±0.15	0-3
W3	13.14±0.29	3.36±0.11	0-1	25.21±2.10	5.15±0.02	1-3
W4	12.99±0.21	2.89±0.43	0-2	25.05±1.17	4.02±0.06	0-3
W5	14.63±0.58	3.06±0.13	0	23.21±0.16	3.53±0.04	1-4
W6	13.08±0.21	3.52±0.35	0-1	28.48±0.35	5.51±0.03	1-3
W7	14.25±0.34	3.32±0.39	0	30.82±1.16	5.81±0.55	1-4
W8	12.69±1.03	3.4±0.14	0-1	27.68±0.53	6.27±0.24	0-4
W10	13.55±0.23	3.08±0.05	0-1	23.66±0.15	5.98±0.25	0-4
S E (m)	0.53	0.23	-	0.96	0.29	-
CD 5%	1.58	0.53-		2.87	0.71	-

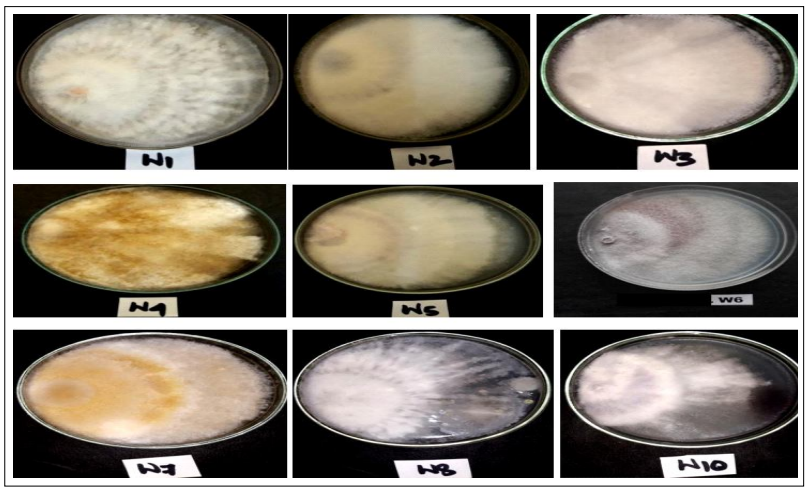


Fig 2: *Fusarium* sp. growth in PDA medium after 7 days of incubation.

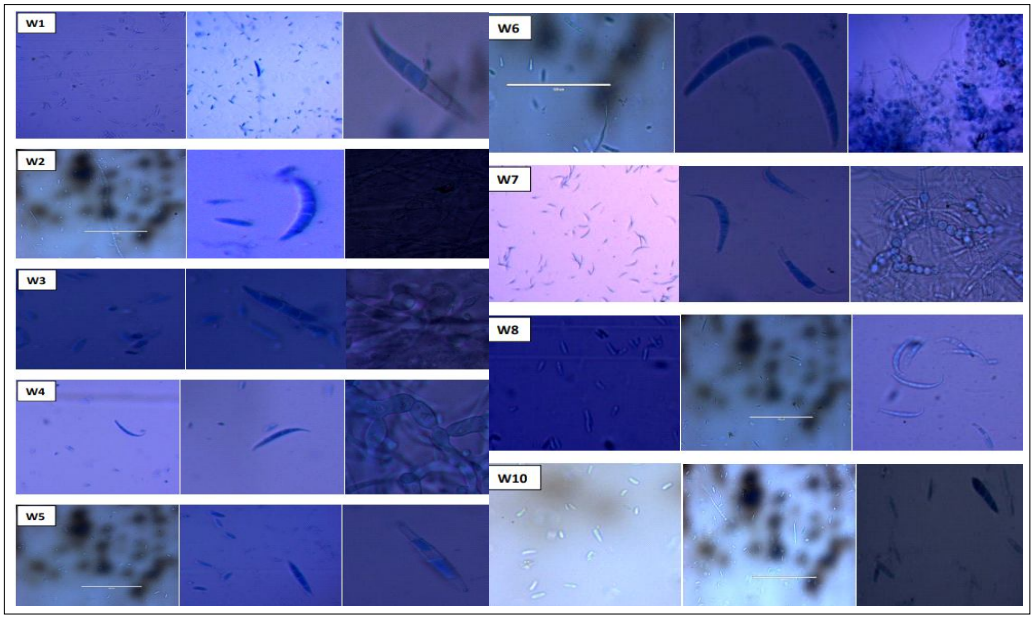


Fig 3: Anamorphic characteristics of Fusarium sp.Presence of macroconidia, microconidia and chlamydospore.

the number of septation of micro conidia ranged from 0-2. The maximum length of macro conidia was observed in W7 (30.82±1.16 µm) and minimum in W1 (21.57±0.52 µm). In case of breadth of macro conidia, the maximum breadth was noticed on W8 (6.27±0.24 µm) and minimum in W5 (3.53±0.04ìm) with an average of 0-4 septa. The results of this study match with Belabid *et al*. (2004) who reported a single race of *F*. *oxysporum* f. sp. *lentis* in Algeria where macro and micro conidia showed great variation in terms of size, shape and septation.

Virulence (Pathogenicity) of the isolates

Pathogenicity test by Weideman and Wehner (1993) showed that there was a significant variation in disease severity on lentil variety Maitri with nine different isolates (Fig 4, Table 3). The isolate W7 produced highest PDI (93.33%) and minimum disease severity was noticed on W1 (75.00%). Among the nine isolates it was found that, the isolates W7 produced maximum PDI and was highly virulent isolate. According to disease severity the isolates were grouped in following descending order W7>W8>W2> W3>W5>W4=W6=W10>W1. Considering the disease severity of all the isolates, the maximum PDI producing (most virulent) isolate was W7 (sample collected from farmer's field of Berhampore, Murshidabad).

There was a significant variation in disease severity among the nine isolates of *Fusarium* on lentil variety HUL-57 with a few exceptions. The isolate W1, W4, W7 showed maximum disease incidence (100%) and minimum disease severity was noticed on W5 (86.66%). Among the nine isolates it was found that, the isolates W1, W4, W7 produced maximum PDI and were highly virulent isolate. The degree of disease severity on the variety BM6,the isolate W1, W2, W3, W6, W7 and W10 showed maximum disease incidence (100.00%) whereas minimum disease severity was noticed on W4(93.10%). Though *F. oxysporum* f. sp. *lentis* has a narrow host range, it does exhibit great variability in virulence/aggressiveness as has been reported earlier (Mondal *et al*., 2020).

Molecular variability of different isolates of *Fusarium sp.*

Identification of molecular variability was performed by sequence analysis of rDNA-ITS region of each isolate for confirmation of molecular variability of the *Fusarium* isolates.

Molecular identification of *Fusarium sp.* based on ITS

The total size of the ITS1 and ITS4 regions, including the 5.8S rDNA gene of the isolates studied varied from 371 to 568 bp (Fig 5). The sequences were identified and deposited in NCBI GenBank, phylogenetic tree was also constructed using Neighbor-Joining (J) method of mathematical averages (UPGMA). W2, W3, W4, W6, W7, W8, W10 were identified as *Fusarium oxysporum* with the accession number MN758599, MN758600, W4 MN758601, MN758603, MN758604, MN758605, MN818591 respectively and W1 and W5 as *Fusarium equiseti* MN758598 and MN758602 respectively (Table 4).

Table 3: Pathogenecity tests of different Fusarium isolates against three susceptible variety of lentil *viz.* MAITRI, HUL-57 and BM-6 under artificial growing condition.

	MAITRI					HUL-57					BM-6				
Isolates	Total plants	Number of germinated plants	% Germination	No. of wilted plants	PDI (%)	Total number of germinated plants	% Germination	No. of wilted plants	PDI (%)	Total number of germinated plants	% Germination	No. of wilted plants	PDI (%)
W1	30	28	93.33 (75.00)	21	75.00 (60.00)	27	90.00 (71.56)	27	100.0 (90.00)	27	90.00 (71.56)	27	100.0 (90.00)
W2	30	30	100.0 (90.00)	26	86.66 (68.61)	27	90.00 (71.56)	25	92.59 (74.21)	27	90.00 (71.56)	27	100.0 (90.00)
W3	30	27	90.00 (71.56)	23	85.18 (67.37)	25	83.33 (65.88)	22	88.00 (69.73)	27	90.00 (71.56)	27	100.0 (90.00)
W4	30	30	100.0 (90.00)	23	76.66 (61.07)	28	93.33 (75.00)	28	100.0 (90.00)	29	96.66 (79.53)	27	93.10 (74.77)
W5	30	29	96.66 (79.53)	19	79.31 (62.94)	30	100.0 (90.00)	26	86.66 (68.61)	28	90.33 (71.95)	27	96.42 (79.06)
W6	30	30	100.0 (90.00)	23	76.66 (61.14)	29	96.66 (79.53)	27	93.10 (74.77)	30	100.0 (90.00)	30	100.0 (90.00)
W7	30	30	100.0 (90.00)	28	93.33 (75.00)	28	93.33 (75.00)	28	100.0 (90.00)	30	100.0 (90.00)	30	100.0 (90.00)
W8	30	30	100.0 (90.00)	27	90.00 (71.57)	28	93.33 (75.00)	27	96.24 (78.76)	28	90.33 (71.95)	27	96.42 (79.06)
W10	30	30	100.0 (90.00)	23	76.66 (61.14)	27	90.00 (71.56)	26	96.29 (78.91)	27	90.00 (71.56)	27	100.0 (90.00)
S.E(m)±					1.30				2.13				1.68
C.D at 5%					3.89				7.37				4.48

Figures in parenthesis are angular transformed values.

Table 4: Identification of Fusarium isolates based on ITS sequences through BLASTN search of the GenBank database.

Isolate	Length of ITS region sequenced	Gene bank accession number	Most closely related organisms			
			ITS identification	Accession description %	Gene identity	% Query coverage
W1	371	MN758598	F. equiseti	MK290391	97.36	98.00
W2	560	MN758599	F. oxysporum	MT032618	97.75	86.00
W3	374	MN758600	F. oxysporum	KX655586	99.45	97.00
W4	568	MN758601	F. oxysporum	KU258683	97.48	97.00
W5	560	MN758602	F. equiseti	MN759075	99.09	91.00
W6	567	MN758603	F. oxysporum	MN272281	98.92	92.00
W7	567	MN758604	F. oxysporum	MN567668	98.23	97.00
W8	563	MN758605	F. oxysporum	MN759069	98.75	99.00
W10	562	MN818591	F. oxysporum	MN759070	98.75	99.00

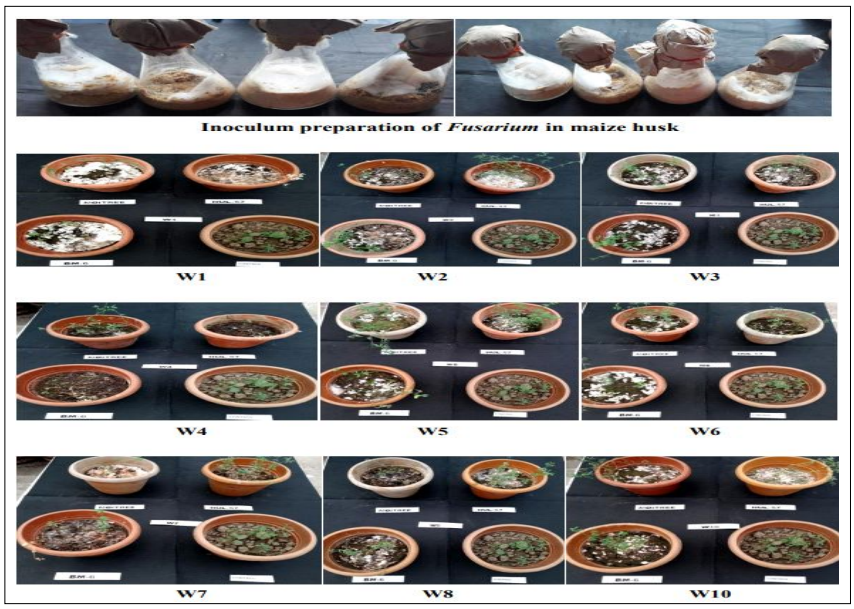


Fig 4: Pathogenicity test using three varieties of lentil *viz*. Maitri, HUL-57 and BM-6 against 9 isolates of *Fusarium* sp.

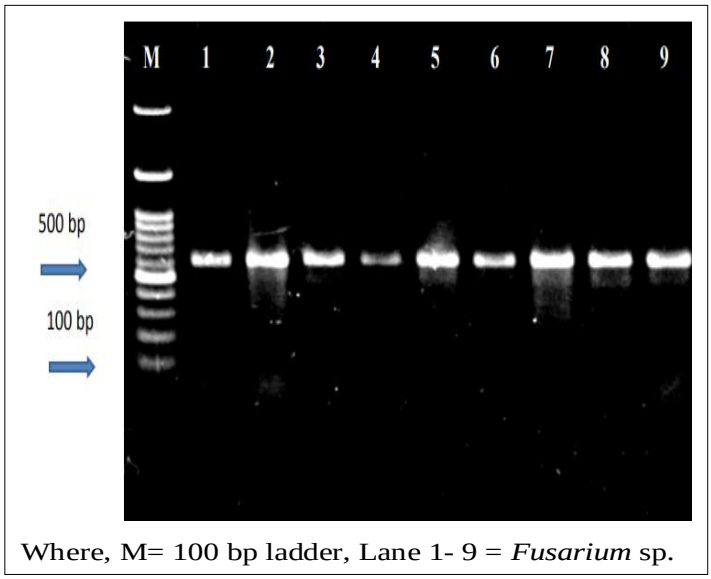
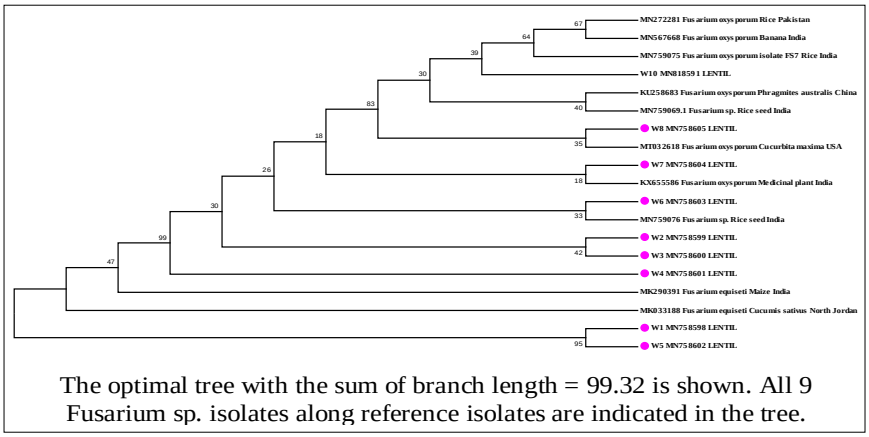


Fig 5: Amplified PCR product of the isolated Fungal DNA ~600 bp visualized in 1% agarose gel.

Table 5: Identity matrix of *Fusarium* isolates considered in this study with other Indian and World isolates based on ITS sequence analysis.

	W1	W2	W3	W4	W5	W6	W7	W8	W10	MK290391	MK033188	MT032618	KX655586	KU258683	MN759075	MN272281	MN567668	MN759076	MN759069
W1 MN758598	ID	92.5	91.8	92.8	92.5	92.0	92.0	92.0	92.0	97.1	97.1	91.8	92.0	92.0	92.0	91.8	91.8	92.0	92.0
W2 MN758599	92.5	ID	99.8	99.8	96.8	100.0	100.0	100.0	100.0	94.0	93.7	99.7	100.0	100.0	100.0	99.7	99.7	100.0	100.0
W3 MN758600	91.8	99.8	ID	99.6	96.5	99.7	99.7	99.7	99.7	93.7	94.0	99.4	99.7	99.7	99.7	99.4	99.4	99.7	99.7
W4 MN758601	92.8	99.8	99.6	ID	96.8	100.0	100.0	100.0	100.0	94.0	94.3	99.7	100.0	100.0	100.0	99.7	99.7	100.0	100.0
W5 MN758602	92.5	96.8	96.5	96.8	ID	96.8	96.8	96.8	96.8	94.3	94.3	96.6	96.8	96.8	96.8	96.6	96.6	96.8	96.8
W6 MN758603	92.0	100.0	99.7	100.0	96.8	ID	100.0	100.0	100.0	94.0	94.0	99.7	100.0	100.0	100.0	99.7	99.7	100.0	100.0
W7 MN758604	92.0	100.0	99.7	100.0	96.8	100.0	ID	100.0	100.0	94.0	94.0	99.7	100.0	100.0	100.0	99.7	99.7	100.0	100.0
W8 MN758605	92.0	100.0	99.7	100.0	96.8	100.0	100.0	ID	100.0	94.0	94.0	99.7	100.0	100.0	100.0	99.7	99.7	100.0	100.0
W10 MN818591	92.0	100.0	99.7	100.0	96.8	100.0	100.0	100.0	ID	94.0	94.0	99.7	100.0	100.0	100.0	99.7	99.7	100.0	100.0
MK290391	97.1	94.0	93.7	94.0	94.3	94.0	94.0	94.0	94.0	ID	100.0	94.3	94.0	94.0	94.0	94.3	94.3	94.0	94.0
MK033188	97.1	94.0	93.7	94.0	94.3	94.0	94.0	94.0	94.0	100.0	ID	94.3	94.0	94.0	94.0	94.3	94.3	94.0	94.0
MT032618	91.8	99.7	99.4	99.7	96.6	99.7	99.7	99.7	99.7	94.3	94.3	ID	99.7	99.7	99.7	100.0	100.0	99.7	99.7
KX655586	92.0	100.0	99.7	100.0	96.8	100.0	100.0	100.0	100.0	94.0	94.0	99.7	ID	100.0	100.0	99.7	99.7	100.0	100.0
KU258683	92.0	100.0	99.7	100.0	96.8	100.0	100.0	100.0	100.0	94.0	94.0	99.7	100.0	ID	100.0	99.7	99.7	100.0	100.0
MN759075	92.0	100.0	99.7	100.0	96.8	100.0	100.0	100.0	100.0	94.0	94.0	99.7	100.0	100.0	ID	99.7	99.7	100.0	100.0
MN272281	91.8	99.7	99.4	99.7	96.6	99.7	99.7	99.7	99.7	94.3	94.3	100.0	99.7	99.7	99.7	ID	100.0	99.7	99.7
MN567668	91.8	99.7	99.4	99.7	96.6	99.7	99.7	99.7	99.7	94.3	94.3	100.0	99.7	99.7	99.7	100.0	ID	99.7	99.7
MN759076	92.0	100.0	99.7	100.0	96.8	100.0	100.0	100.0	100.0	94.0	94.0	99.7	100.0	100.0	100.0	99.7	99.7	ID	100.0
MN759069	92.0	100.0	99.7	100.0	96.8	100.0	100.0	100.0	100.0	94.0	94.0	99.7	100.0	100.0	100.0	99.7	99.7	100.0	ID



The optimal tree with the sum of branch length = 99.32 is shown. All 9 Fusarium sp. isolates along reference isolates are indicated in the tree.

Fig 6: The neighbor-joining tree of *Fusarium* isolates based on ITS region sequences.

Sequence analysis of ITS (*Fusarium* sp.) and evolutionary relationship

ITS sequences of *Fusarium* isolates W1-W10 were aligned with the consensus region using CLUSTAL W program. Phylogenetic analysis grouped the *Fusarium* isolates into two clusters, Cluster I include *Fusarium* isolates W2, W3, W4, W6, W7, W8, W10 with a bootstrap support of 47% and cluster II includes *Fusarium* isolates W1 and W5 with a bootstrap support of 95% (Fig 6). Singha *et al*., (2016) has stated that ITS sequence analysis is one of the most accurate methods of revealing taxonomic and phyllogenetic relationships among *Fusarium* complex. Identity matrix of the Fusarium isolates ranged between 91.7% to 100%. Genetic identity of the isolates retrieved from NCBI Gen-bank obtained was 91.8% to 100% (Table 5). A report on the phylogenetic tree by Nirmaladevi *et al*. (2016) based on the ITS sequence analysis revealed four major groups suggesting four major evolutionary lineages of *Fusarium oxysporum* f. sp. *lycopersici* existing in tomato growing regions of India.

CONCLUSION

The present study reports wide distribution of lentil wilt disease in the lentil growing parts of West Bengal, India and showed prevalence of morphologically and genetically diverse isolates of *Fusarium oxysporum* possessing considerable pathogenic variability. Sequence analysis of rDNA-ITS region along with morphological and pathogenic data for characterization of the isolates greatly enhanced the understanding of the variability within this important fungus. This could ultimately benefit for the management of wilt disease through host plant resistance.

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Conflict of interest

All authors declare they have no conflict of interest with this article.

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