



First report of *Exserohilum* leaf spot: a unique halophilic pathogen in *Cucumis sativus* in the South Bengal area of India

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Abstract

Cucumber (*Cucumis sativus* L.) is a nutritious domesticated vegetable which belongs to Cucurbitaceae family. India ranks 30th in global cucumber production and its total output is 1,61,000 metric tons annually. In the investigation a different kind of leaf spot disease was observed on 2 months old cucumber leaves samples from cucumber fields in Baruipur, West Bengal, India (Latitude: N 22° 22.262', Longitude: E 88° 24.5086'). The spots appeared were large, greyish brown in colour surrounded by a yellow to brownish halo, measuring 2 × 1 cm in average. These lesions expanded gradually from circular to angular spots ultimately resulting in necrosis and defoliation. The isolated fungus showed greyish black to dark olive mycelial growth. Microscopic characterization revealed straight to slightly curved conidia and distoseptate with a distinctly protruding basal hilum. Through microscopic observation and ITS sequencing the organism was identified to be *Exserohilum rostratum*. *E. rostratum* was previously known for causing blight disease in maize as well as human immune system diseases. *E. rostratum*, *E. longirostratum* and *E. mcginnisii* are identified as human pathogens. Koch's postulates were carried out and distinct symptoms were observed in cucumber. As because there is no such record, this is the first time the host pathogen combination is separated in India.

Keywords Unique pathogen · *Exserohilum rostratum* · *Cucumis sativus* · Cucurbitaceae · Leaf spot disease

Introduction

India suffers a huge loss of agricultural produce due to bacterial and fungal attacks. Identification of these pathogens can help in prevention of these attacks. With the passage of time, new host-pathogen relationships are discovered which is mainly due to the ability of the pathogen to shift from one host to another. This ability to infect more than one host depends on the evolutionary history of both pathogen and its host. By investigating the host range variation within a family and using phylogenetic studies one can find out the frequency of host jump events in the course of evolution in that family (Navaud et al. 2018).

Cross kingdom pathogens are those capable of infecting hosts belonging to different kingdoms. For example, *Burkholderia cepacia* and *Aspergillus flavus* are potential phytopathogens who have evolved to infect humans.

B. cepacia causes fibrosis and *A. flavus* causes fungal keratitis in humans (Jones et al. 2001; Krishnan et al. 2009). To be a cross kingdom pathogen it should evolve to a level where it can survive in different hosts. Cross kingdom gene transfers are believed to be the source of emergence of these cross kingdom pathogens. When searched for prokaryotic derived HGTs in 60 fully sequenced fungal genomes, 713 transferred genes were detected (Marcet-Houben and Gabaldón 2009). This reveals that cross kingdom HGTs must have played a role in evolution of fungi. Pathogen host jumps have become a major source of new infections in plants as well as humans.

Exserohilum is a dermatiaceous fungi which belongs to the family Pleosporaceae and is known to cause blight in plants. *Exserohilum* is an anamorphic fungi having the teleomorphic or sexual reproductive stage i.e. *Setosphaeria*. *Exserohilum* reproduces by the formation of conidia with typically 4–12 septa (Bunkoed et al. 2014). The presence of a protruding hilum at the base of the conidia is an identifying feature of *Exserohilum*. The conidia are multi-distoseptate, fusiform, cylindrical or obclavate, straight or curved (Hernández-Restrepo et al. 2018).

Exserohilum has emerged to be a potential cross kingdom pathogen. Its relation with human diseases is well

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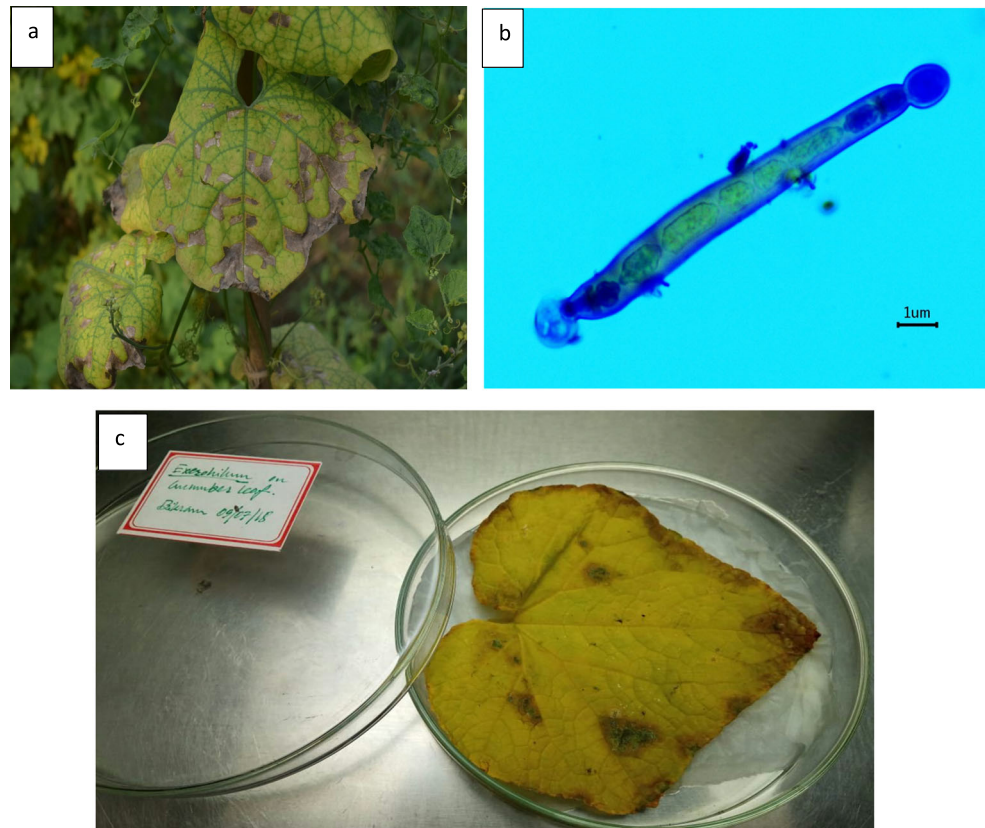
Table 1 Details of isolates included in phylogenetic analysis

Species	GenBank Accession	Isolate	Source	Geographical region
<i>Exserohilum rostratum</i>	MH779469	C1	<i>Cannabis sativa</i> L.	USA: North Carolina
<i>Exserohilum rostratum</i>	MN337265	BDAM_st18	<i>Cucumis sativus</i>	India: South Bengal
<i>Exserohilum rostratum</i>	MK937620	Y9511	–	China
<i>Setosphaeria rostrata</i>	KT274699	61A	<i>Chloris gayana</i>	Argentina: Buenos Aires
<i>Setosphaeria rostrata</i>	KT265248	CBS 134641	Collected genotyped isolates	Chile
<i>Bipolaris rostrata</i>	MG551564	1709	–	India: New Delhi
<i>Setosphaeria rostrata</i>	MH178696	IUB:B.Whitaker:OTU27	<i>Panicum virgatum</i>	U.S.A
<i>Setosphaeria rostrata</i>	MH201152	UAS BW 10176	<i>Triticum aestivum</i>	India: Uttar Pradesh
<i>Exserohilum mcginnisii</i>	MH818405	AK-MSR37	Mangroves	India
<i>Exserohilum mcginnisii</i>	AF081453	ATCC 60408	Human nasal sinus	Arizona
<i>Exserohilum mcginnisii</i>	MH864799	CBS 128056	–	Netherlands
<i>Exserohilum mcginnisii</i>	MN218570	BMU00224	–	China
<i>Exserohilum mcginnisii</i>	NR_157456	CBS 325.87	Human nasal polyp	Arizona
<i>Exserohilum longirostratum</i>	KF725822	E5	–	India
<i>Exserohilum longirostratum</i>	KF725828	E11	–	India
<i>Exserohilum longirostratum</i>	AF163064	7728	–	China
<i>Exserohilum longirostratum</i>	MH864797	CBS 128054	<i>Acacia mellifera</i> subsp. <i>detinens</i>	Namibia
<i>Exserohilum holmii</i>	MH319027	L-2718/16	Human	India
<i>Exserohilum holmii</i>	MH319028	L-2963/16	Human	India
<i>Exserohilum holmii</i>	MH319024	L-2204/16	Human	India
<i>Exserohilum neoregeliae</i>	NR_157457	CBS 132832	<i>Neoregelia carolinae</i>	Japan
<i>Exserohilum neoregeliae</i>	AB607957	IM201-E	<i>Neoregelia carolinae</i>	Japan
<i>Exserohilumneoregeliae</i>	KT265255	CBS 132833	<i>Neoregelia carolinae</i>	Japan
<i>Exserohilum minor</i>	AF163065	81100b	–	China
<i>Setosphaeria minor</i>	AF071341	ATCC 62323	<i>Dactyloctenium aegyptium</i>	Australia
<i>Exserohilum minor</i>	NR_158419	BRIP 14616	<i>Dactyloctenium aegyptium</i>	Australia
<i>Exserohilum turcicum</i>	NR_163537	CBS 690.71	<i>Zea mays</i>	Germany
<i>Exserohilum turcicum</i>	MK332496	RS_82	<i>Spilanthes paniculata</i>	India: Andhra Pradesh
<i>Exserohilum turcicum</i>	AF163067	94/1823	–	China
<i>Exserohilum turcicum</i>	MK800000	I25	<i>Zea mays</i>	Argentina
<i>Setosphaeria turcica</i>	KJ922737	SHG1303	<i>Sorghum bicolor</i>	China: Suihua Helongjiang
<i>Setosphaeria turcica</i>	KJ922738	SHY1309	<i>Zea mays</i>	China: Suihua Helongjiang
<i>Setosphaeria monoceras</i>	AF071340	DAOM 208988	–	Canada
<i>Setosphaeria monoceras</i>	KR263036	EX 6	<i>Echinochloa crus-galli</i>	Iran
<i>Setosphaeria monoceras</i>	KR263035	EX 5	<i>Setaria</i> sp.	Iran
<i>Exserohilum oryzicola</i>	NR_138225	CBS 502.90	<i>Oryza sativa</i>	Columbia
<i>Exserohilum pedicellatum</i>	AF229478	EEB 1336	–	U.S.A
<i>Exserohilum pedicellatum</i>	NR_157425	CBS 322.64	<i>Triticum aestivum</i>	USA
<i>Bipolaris maydis</i>	NR_138224	CBS 136.29	<i>Zea mays</i>	Japan
<i>Bipolaris maydis</i>	KM230389	C4	<i>Zea mays</i>	U.S.A

documented (Adler et al. 2006). A major outbreak took place in the United States in 2012 which resulted in 64 deaths and 753 cases in total. This outbreak included meningitis, paraspinal/spinal infection and peripheral joint infection. *Exserohilum rostratum* was identified to be the predominant fungus in patients (60.4%) followed by *E. longirostratum* (6.3%) and *E. mcginnisii*.

Remaining 31% were unidentified species. The source of these infections were found to be contaminated preservative-free methylprednisolone acetate (MPA) steroid injections (Katragkou et al. 2014). Cases of fungal keratitis caused by *E. rostratum* was reported in India and Thailand in 2012 and 2019 respectively (Joseph et al. 2012; Chaidaroon et al. 2019).

Fig. 1 Cucumber leaf showing angular leaf spot (a), multiseptate conidia with protruding hilum (b), cucumber leaf inoculated with spores showing angular spots (c)



Exserohilum is also a phytopathogen which causes leaf spot on grass (Brunings et al. 2009). It is also known to cause infections in both monocotyledonous and dicotyledonous plants which includes leaf spot disease in pineapple plants (Luo et al. 2012), rice brown spot (Toher et al. 2016), northern corn leaf blight (Bashir et al. 2018), banana leaf spot (Shan-Hai et al. 2011) and leaf spot on bottle gourd (Choudhary et al. 2018). Different parameters like relative humidity, rainfall and temperature were shown to favour maize infection by *Exserohilum* (Nwanosike and Mabagala 2017). Apart from its pathogenicity, its potential as a biocontrol agent is also verified (Tsukamoto et al. 1997; Kadir et al. 2007).

The main objective of this paper is to report a new host pathogen relationship in the South Bengal area of India. This new host pathogen interaction constitutes of *Exserohilum* as pathogen and *Cucumis sativus* L. (cucumber) as host. This indicates the potential of *Exserohilum* as a competent plant pathogen and raises concern regarding cross kingdom infections.

Materials and methods

Pathogen isolation from cucumber plants

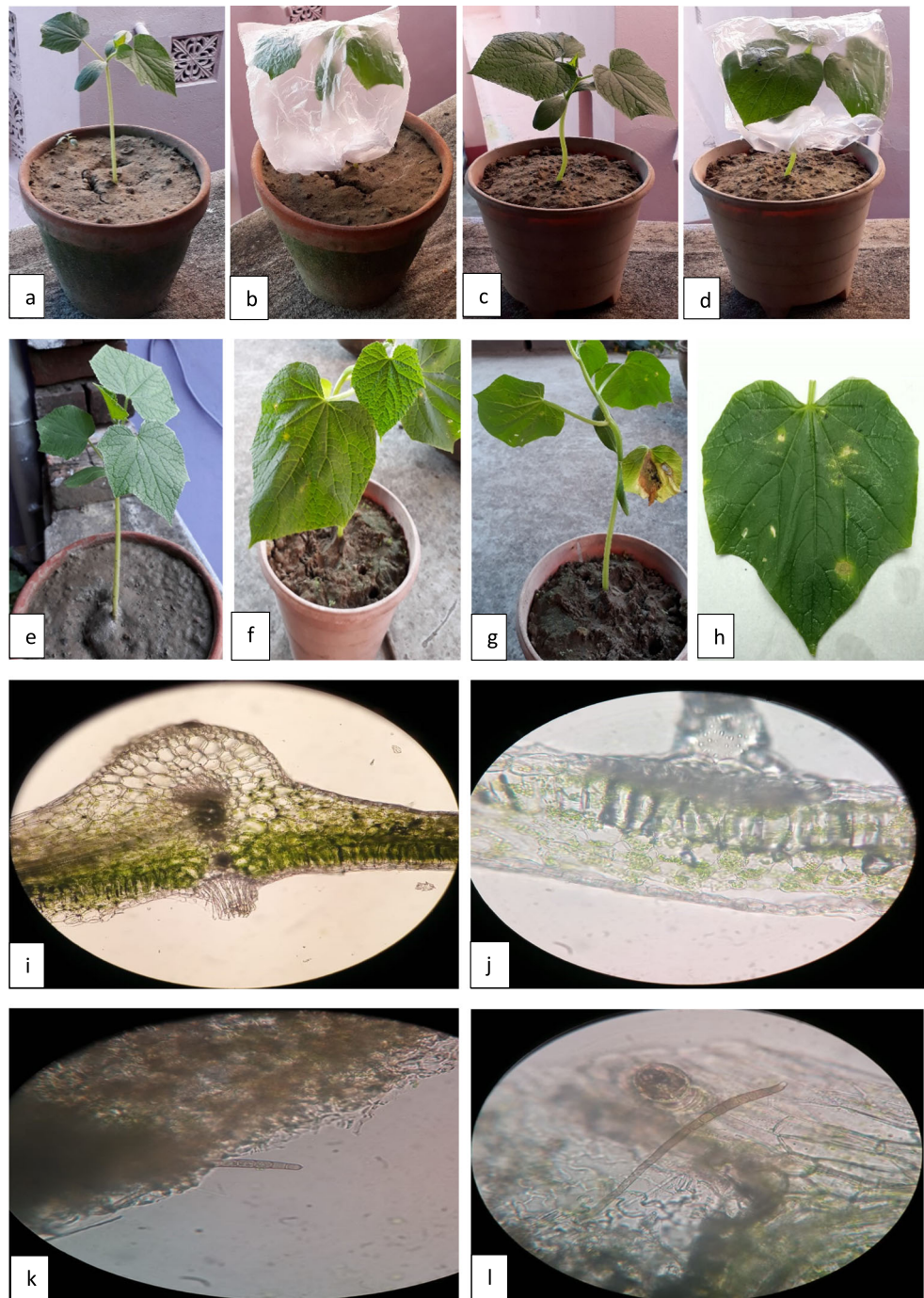
The pathogenic fungus was isolated from diseased leaf sample collected from Baruipur in 2018. These were collected in air-tight zip lock bags and stored at 4 °C.

The diseased tissue was randomly selected, cut into 1 × 1 cm pieces by flame sterilized forceps and washed in sterile water, dipped in 75% ethanol for 2–3 s, followed by HgCl₂ solution (0.1% HgCl₂ (w/v) in sterile distilled water) wash for 1 min. These leaf pieces were washed in sterile water and blotted dry with sterilised blotting paper to remove excess water. 3–4 pieces of surface sterilized leaf pieces were placed onto Potato dextrose agar (PDA) plates supplemented with streptomycin (HIMEDIA Streptomycin sulphate (TC035-5G) of 100 ppm concentration) and were incubated at 28 °C for 48 h for appearance of colonies. These were subcultured by inoculating fresh PDA slants with added streptomycin of same concentration and incubated at 28 °C until sporulation occurred followed by 4 °C storage.

Morphological characterization

The fungus was transferred to fresh Potato Dextrose Agar (PDA) plates and incubated for 10 days at 30 °C in a B.O.D. incubator. The colony growth, colour, conidial shape and size were examined in a Dewinter compound light microscope. Spore size were measured on the average of 100 spores and scale bars were added with the photographs taken during the morphological characterization.

Fig. 2 plant inoculated with distilled water (**a, b**), plant inoculated with spores (**c, d**), control shows no angular leaf spots (**e**), leaves inoculated with spores show angular leaf spots (**f, g, h**), cross-section of leaves inoculated with distilled water (**i, j**) and sores (**k, l**)



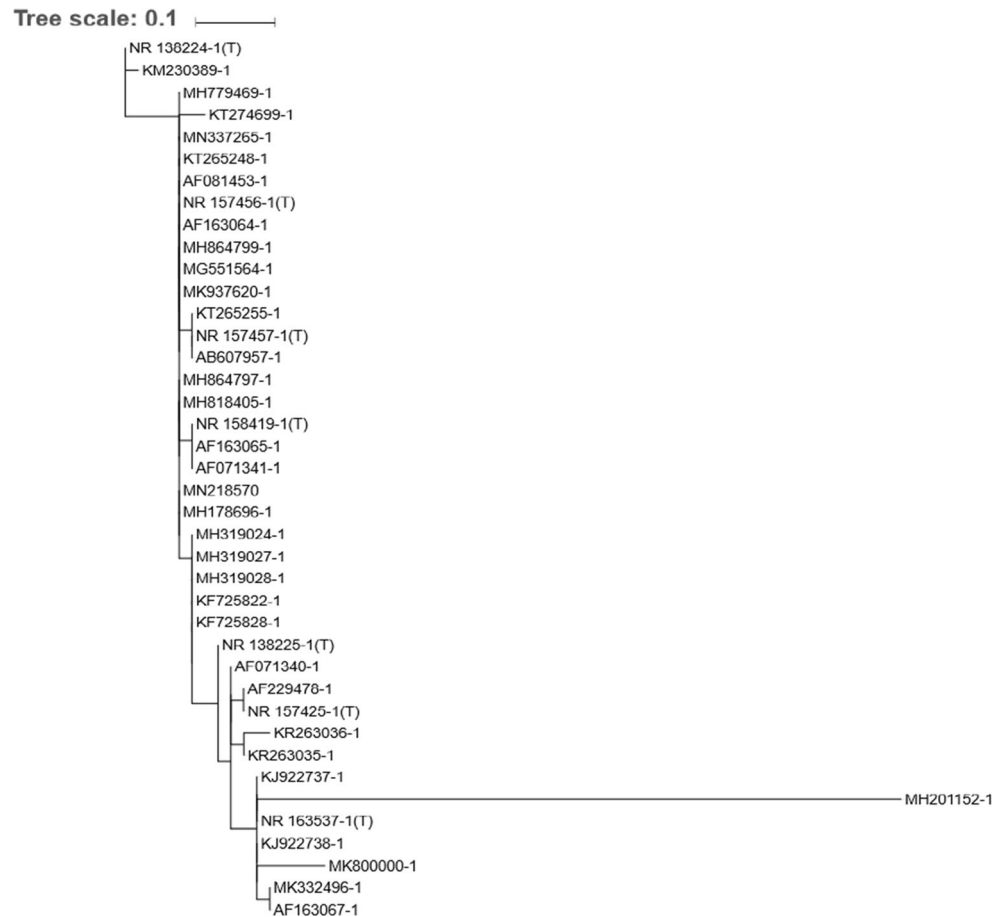
Pathogenicity tests

The in vitro pathogenicity test was conducted using single cucumber leaf inoculated in Petri plate by spraying with conidial suspension of the isolate. The conidial suspension was prepared by gently scraping *Exserohilum* spores from the surface of media and then adding it to 10 ml sterile distilled water. The conidial concentration used for the test was 4.2×10^6 spores/ml. Negative control comprised of a leaf with no added inoculum and 100 μ l of spore suspension was added to each of the experimental leaves

in the form of droplets. These leaves were incubated in a covered sterilized Petri plate with adequate moisture. Lesions appeared in the experimental leaves after 3 days of inoculation.

The in vivo pathogenicity test was conducted using two sets of plants, first set was sprayed with conidial suspension (same as used for in vitro test) and the second set was sprayed with sterilized water. Both the upper and lower surface of each leaf of the healthy plants were sprayed 3–4 times and then these plants were carefully covered with UV-sterilized transparent plastic bags to ensure high relative humidity without causing any mechanical

Fig. 3 Phylogenetic tree inferred using PhyML+SMS software and viewed in iTOL. Bayesian posterior probability is >0.91 for every node. Type strains are indicated as (T)



damage to the plant. Plants were incubated at 30 °C with a natural photoperiod. Number of plant replicates taken in each set were ten.

Leaf sectioning was observed via light microscopy (Dewinter Excel Microscope) to detect pathogen protrusion through the cross sectional area. Re-isolation of fungus from the inoculated leaves was conducted to prove Koch's postulates. Re-isolated fungus and the fungus isolated from the field plant was compared via microscopic observation.

18S rRNA and ITS (Internal transcribed spacer) Sequencing

18S rRNA and ITS Sequencing was performed for the identification of the fungus in this study by using universal primer (Wang et al. 2014).

Phylogenetic analysis

ITS sequences of 38 different species of *Exserohilum* including the isolated sequence of *E. rostratum* MN337265 was used for phylogenetic analysis (Table 1). Multiple sequence alignments were conducted by MAFFT (Kato and Standley 2013), followed by Gblocks for aligned sequence optimization (Talavera and

Castresana 2007). PhyML+SMS software was used for design of phylogenetic tree in which aBayes was the selected method for bootstrapping (Lefort et al. 2017; Guindon et al. 2010; Lemoine et al. 2018). The produced phylogenetic tree bearing the evolutionary distances among *Exserohilum sp.* isolates was viewed in Newick display with the help of iTOL (Junier and Zdobnov 2010; Letunic and Bork 2019).

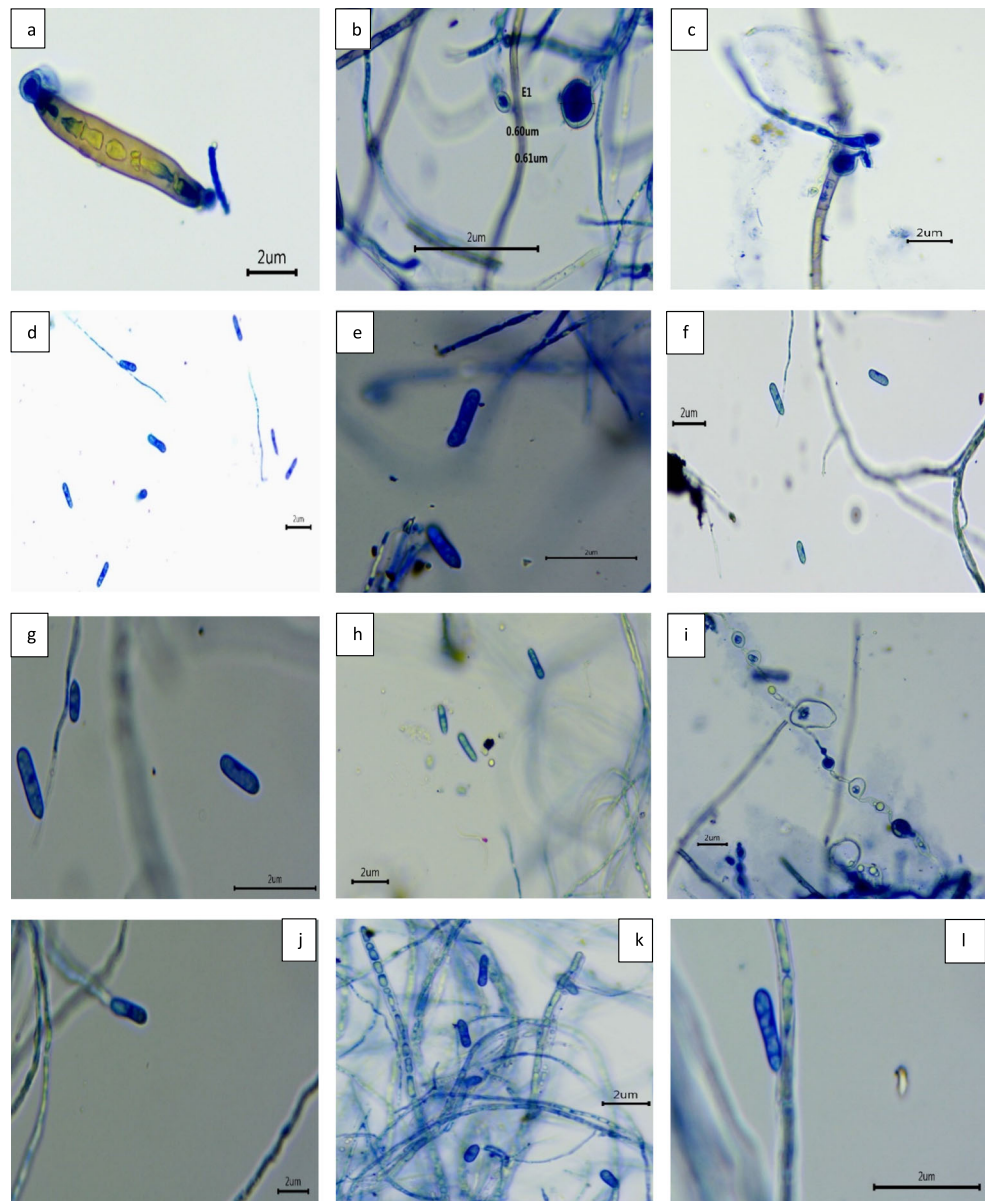
Sporulation study in relation to salt stress

The fungal pathogen was cultured in salt stress induced media. These were grown in PDA using 1%, 2%, 3%, 5%, 7% and 10% NaCl concentrations and incubated for 7 days at 35 °C. This was observed by lactophenol cotton blue stained light microscopy (Dewinter microscope digital camera, DIGI510, 5.1 MP 1/ 2.5 "CMOS sensor).

Influence of culture media on growth characteristics

Six millimeter discs of test fungi obtained from 7 days old pure cultures were transferred at the centre of sterile Petri plates containing four different media: Potato dextrose agar (PDA)(HIMEDIA M096, suspend 39 g in 1000 ml distilled water), Czapek Dox agar (CZA)(HIMEDIA M075, suspend 49.01 g

Fig. 4 0% salt concentration: conidia (a), presence of chlamydospore (b), gametangial contact (c). 2% salt concentration: conidia in 40x (d) and 100x (e). 3% salt concentration: conidia in 40x (f) and 100x (g). 5% salt concentration: conidia in 40x (h), presence of oil/lipid bodies and intercalary chlamydospores (i), conidia in 100x (j). 7% salt concentration: conidia and thick walled mycelia in 40x (k) and 100x magnification (l)



in 1000 ml distilled water), Oat meal agar (OMA)(HIMEDIA M397, suspend 72.5 g in 1000 ml distilled water) and Malt extract agar (MEA)(HIMEDIA M1913, suspend 61 g in 1000 ml distilled water). The petri plates were then incubated at 25 °C and observed regularly. Sporulation was observed via staining under microscope.

Results

Microscopic examination

Cottony white colonies appeared after 48 h of incubation of isolated fungus. Colony eventually turns grayish brown to black after 7 days of incubation. Mycelia showed brown colouration

under microscope. Conidia were straight or slightly curved ellipsoidal to fusoid, with typically 4–12 distosepta as revealed by lactophenol cotton blue staining by light microscopy (Fig. 1a, b). The conidia were characterized by the presence of a strongly protruding beak shaped basal hilum. The septa above the hilum is much thicker than that of the intercalary ones and retains dark stain. Apart from conidial structures there was no other kind of spores. Spore size was measured to be 40–160 μm in length and 12 μm in breadth. In few cases the fungus was found to undergo sexual reproduction by means of gametangial contact.

Pathogenicity tests

The in vitro pathogenicity test showed irregular shaped brown outlined olive greenish to grayish brown spots scattered

Table 2 Mycelial growth, colony characteristics and sporulation pattern of test fungi on four different culture media

Media type	Colony diameter (mm)			Colony character		Zonation	Sporulation
	2 days incubation	4 days incubation	6 days incubation	Texture	Surface colour		
CZA	27	59	75	Cottony, raised in centre.	Buff coloured colony with creamy white margins.	Concentric	Low
OMA	27	58	72	Cottony, raised in centre.	Greyish black to black colony with white cottony margins and centre.	Concentric	Heavy
MEA	25	55	71	Cottony, raised in centre.	Cottony white centre, dark olive green to black colony with light coloured margin.	Concentric	Moderate
PDA	15	36	48	Cottony, raised in centre.	Black colony with light cottony centre and margin.	Concentric	Heavy

throughout the leaf surface and brown spots along the periphery (Fig. 1c). The control sample showed no spots after the incubation.

Under in vivo treatment, inoculated leaves of cucumber plants showed similar spots with distinct yellow hallow 7 days after spray application. Leaves sprayed with sterilized water showed no change in appearance. The pathogen isolated from the infected leaves showed ellipsoidal conidia with truncated hilum as observed by light microscopic study of field plant isolated fungus (Fig. 2).

Sequencing

ITS sequencing data via BLAST revealed 98.94% sequence similarity with *Exserohilum rostratum* strain AUMC 14373 ITS sequence. To specify the pathogen isolated from *Cucumis sativus*, lactophenol cotton blue staining followed by light microscopy (Dewinter microscope digital camera, DIGI510, 5.1 MP 1/ 2.5 "CMOS sensor) was conducted. Lactophenol cotton blue stained fungal conidia via light microscopy revealed darkly pigmented basal and distal septa which characterise *E. rostratum*.

Phylogenetic analysis

Exserohilum rostratum (MN337265) is evolutionally more diverged from *Bipolaris maydis* (NR_138224) as compared to *Setosphaeria rostrata* (KT 265248). The most diversified among all the 40 selected fungal species is *Setosphaeria rostrata* (MH201152) having *Triticum aestivum* as the host, found in Uttar Pradesh, India. The two *Setosphaeria monoceras* (KR263036 & KR 263035), originated from Iran has more similarities as compared to the Indian/ American species listed (Fig. 3).

Influence of different salt concentrations on sporulation

The pathogen showed no sporulation and very scanty growth at 1% and 10% salt concentration respectively. Sporulation was observed at 2%, 3%, 5%, 7% salt concentrations via lactophenol cotton blue staining followed by light microscopy (Fig. 4). Under different NaCl concentration the mycelia possesses few special structures. Chlamydospores were observed distinctly in 5% NaCl concentration. With the increasing salt concentration conidial structure was significantly altered. Arthroconidia was found when grown under 2%, 3%, 5% and 7% salt concentration. Number of spores increases but they were greatly reduced in size, ellipsoidal with slightly appressed middle portion. All the spores contained granular structures when grown under salt stress.

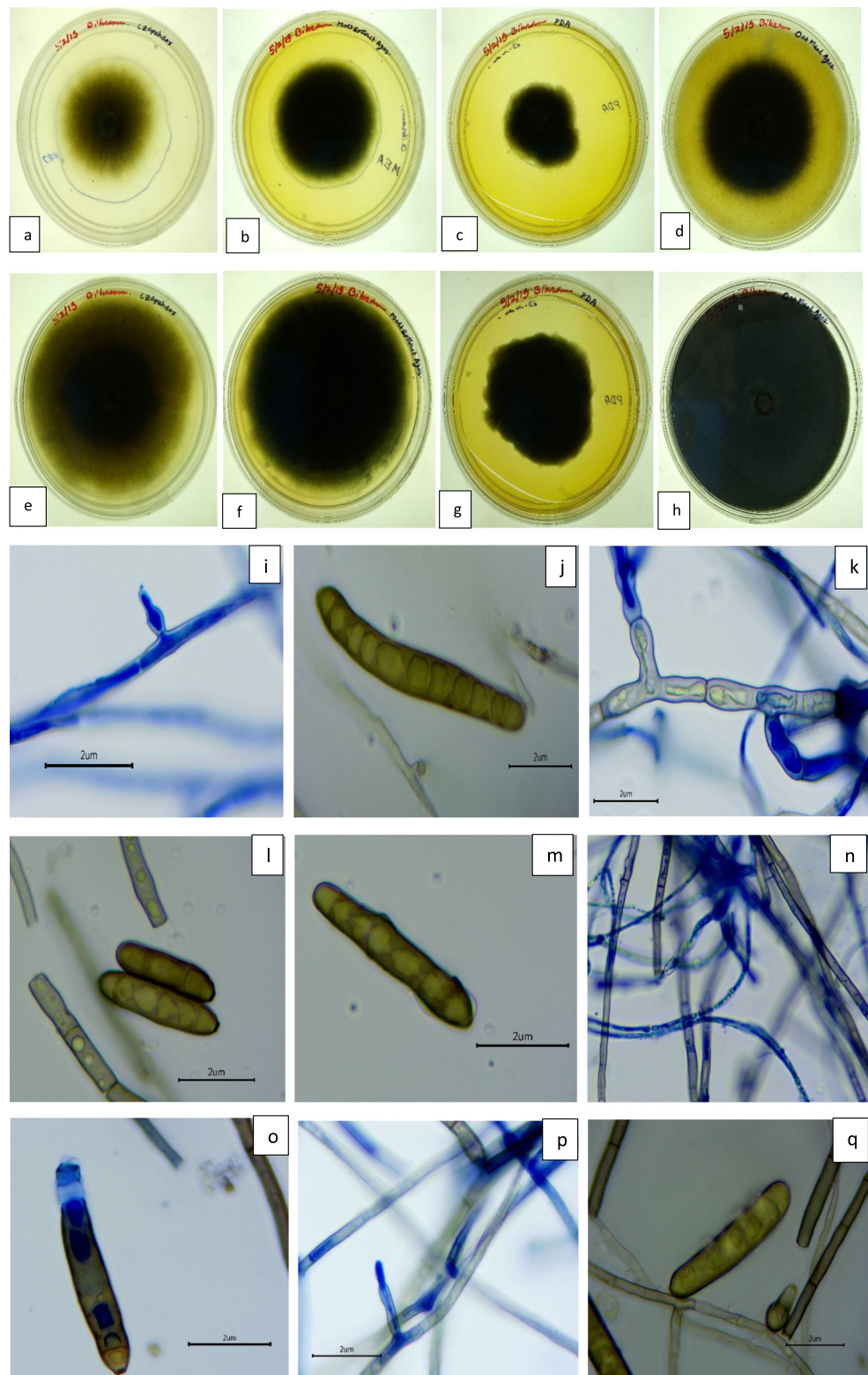
Influence of culture media on growth, colony character and sporulation

All three culture media (PDA, CZA, OMA and MEA) supported the growth of test fungi to various degrees. Out of all these media, the test fungi showed maximum mycelial growth on CZA (75 mm) after 6 days of incubation period (Table 2). In both OMA and MEA, the test fungi showed good growth (72 mm and 71 mm respectively) whereas the least growth was observed in PDA (48 mm). The mycelial colour varied greatly in different media which was further observed and tabulated. Despite of the poor mycelial growth in comparison with other culture media PDA shows early and heavy sporulation (Fig. 5).

Discussion

Disease caused by pathogenic fungus is a major limiting factor in order to produce healthy cucumber plant and increasing the yield. During our current study it has been observed that the

Fig. 5 Colony of test fungi in CZA, MEA, PDA and OMA respectively after 4 days of incubation (**a, b, c, d**) and 6 days of incubation (**e, f, g, h**). Mycelia and spore structures in CZA (**i, j**), MEA (**k, l, m**), PDA (**n, o**) and OMA (**p, q, r**) respectively



whole cucumber field was severely affected by leaf spot disease. Plants were mostly drooping down and under the threat of rapid defoliation. *Exserohilum rostratum* (Drechs. ex.type of *E. macginnisii*) Leonard and Suggs was identified as the

causal organism for the destructive leaf spot disease based on morphological characteristics (Ahmadpour et al. 2013; Shan-Hai et al. 2011). The ITS sequence has been already submitted in GenBank with accession number MN337265 and that

sequence has highest similarity with other *E. rostratum* ITS sequences. Koch's postulates proved the occurrence of the disease in cucumber plant. Microscopic observation showed conidial growth in the mesophyll layer of the infected leaf and partial necrosis of plant tissue. *E. rostratum* was previously known to cause leaf spot disease in bottle gourd of Cucurbitaceae family (Choudhary et al. 2018). But this is for the first time this host pathogen interaction is found in India where the host plant is *Cucumis sativus* L. and the pathogen is *E. rostratum*. In order to characterize the isolated pathogenic strain of *E. rostratum* and to check its conidia production rate the fungus was further grown in different fungal specific media like OMA, MEA, CZA and PDA (Zhang and Watson 1997). All the substrates shown better mycelial growth than that of in PDA. In case of OMA and Czapekdox media luxuriant mycelial growth found may be due to the presence of adequate nitrogenous compound. MEA facilitate typical mycelial growth and pigmentation due to the presence of mycological peptone (Jorgensen et al. 2015). Heavy sporulation was found in OMA as this particular media is rich in Nitrogen, Carbon, Proteins and nutrient (Murray et al. 2003). PDA and MEA also shows moderate to heavy sporulation as these fungal media also stimulate sporulation (MacFaddin 1985).

As the field area from where the pathogen was isolated is not so far from the coastal region and the biggest mangroves of the world, the salt tolerance of the fungus was checked. The fungus showed mycelial growth even in 10% salt (NaCl) concentration. Different spore morphology found in different salt concentrations which reveals that the pathogen is well adapted to combat salt stress. In lower salt concentration some reproductive structures was found. This fungus undergoes sexual reproduction by means of gametangial contact. In 5% salt concentration chlamyospore was found. Depending upon the environmental condition fungus produces different resting spores. The formation of chlamyospores indicates the ability of the pathogen to survive in adverse environmental condition (Levy 1995). So depending upon the morphological characterization it can be concluded as, *E. rostratum* is a potent phytopathogen which is really tough to control by altering physical factors. Further different biological control methods can be adapted in order to eradicate this destructive plant pathogen.

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