Diplodia mutila as a new pathogen on water hyacinth *Eichhornia crassipes* in Harike wetland

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ABSTRACT

The present publication reports the mycobiota associated with *Eichhornia crassipes* (C Mart.) Solms (Pontederiaceae) infesting the Harike Wetland at Punjab in India and their potential application as a biological control agent based on their pathogenicity to the host plant. Of the 30 fungal isolates recovered from the infested samples of *E. crassipes* only one isolate #19BJSS caused 98% damage or infestation to the leaves during the *in vitro leaf* bioassay while it exhibited 100% plant death during the whole plant bioassay after 168 hours post inoculation (hpi). Morphological studies revealed it to be a member of *Botryosphaeriaceae* family which was further confirmed by Internal Transcribed Spacers (ITS) phylogenetic studies wherein the isolate #19BJSS clustered with *Diplodia mutila*. *Botryosphaeria* species have been predominantly associated with canker and die back diseases of woody plants. This is the first report of occurrence of *Diplodia mutila* #19BJSS as a pathogen of *E. crassipes* and for its potential as a biological control agent for the management of water hyacinth.

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INTRODUCTION

Eichhornia crassipes (C. Mart) Solms, (Pontederiaceae) commonly known as water hyacinth is an aquatic plant world-wide. This plant has been designated as among top 100 invasive species by International Union of Conservation of Nature (IUCN) (Tellez *et al.,* 2008). The rapid growth and proliferation rate of water hyacinth is a global concern since it poses serious environmental and conservation problems having socioeconomic repercussions. It has been recognized as the top ten world's worst weed that has invaded Europe, Africa, Asia and North America (Ray and Hill, 2016).

Water hyacinth in India has become a serious problem in Harikewetland, a Ramsar site created in 1953 due to construction of a barrage at the confluence of the rivers Sutlej and Beas in Punjab, India (Singh *et al*., 2016). Harike wetland is of both national and international importance since it is inhabited by diverse flora and fauna of which many have been red listed (Singh *et al*., 2016). Apart from 400 species of avifauna existing in this area there is a huge concentration of migratory birds in this wetland and hence it declared as a bird sanctuary (Tiwana *et al*., 2008). Another important aspect of Harike wetland is being an important source of water for irrigation and drinking through its feeder canals in the states of Punjab and Rajasthan apart from ground water recharging. Presently, approximately 40% of this wetland has been infested by water hyacinth, which causes water loss due to excessive evapotranspiration rate apart from several waterborne disease downstream (ICID report 2002; Matt McDonald Report, 2015). Thus there is a need to revive this water body by suppressing the growth of water hyacinth.

Chemical methods of controlling *Eichhornia* in Harike wetland is not recommended as the water from its feeder canals is being used for drinking as well as irrigation. Mechanical removal has been carried out several times but this is successful only for a short duration since water hyacinth resurges (Ndimele *et al.*, 2011). Biological control of the weeds

using microorganisms is being sought as a cost effective, more benign and effective strategy to control pests. Of the scores of fungal pathogens which attack water hyacinth some promising ones which have the potential to be used as a biological control agent *Cercospora piaropi*, *Alternaria eichhorniae*, *Alternaria alternata*, *Myrothecium roridum* and *Rhizoctonia solani*. Some of these have been tested under controlled conditions and have reportedly reduced the biomass of water hyacinth (Martyn and Freeman 1978; Charudattan *et al,*. 1985; Shabana *et al*., 1995). The innundative or bioherbicidal approach is a well-establishedapproach and many mycoherbicides have been developed and commercialized since mass production of the biological control agent is easily achievable using fermentation process (Saxena, 2015). Hence fungal pathogens offer opportunities to be developed as bioherbicides for the management of *Eichhornia crassipes*.

Fungi associated with *Eichhornia crassipes* have been studies in the Amazon basin and has been prospected for its possible use as mycoherbicide (Evans and Reeder, 2001) and using insects (Coetzee *et al*, 2011). However, very limited information exists on the mycobiota associated with *Eichhornia crassipes* for its prospective use as a biological control agent in Indian aquatic bodies. Nag Raj and Ponnappa (1970) first reported the blight of water hyacinth caused by *Alternaria eichhorniae*. Subsequently an isolate of *Alternaria alternata* (Fr.) Keissler from Haryana (Aneja and Singh, 1989), Tamil nadu and Kerala (MohanBabu *et al.,* 2003) have been tested for the potential as a mycoherbicide to control *Eichhornia crassipes*. *Fusarium pallidoroseum* and *Myrothecium advena* have also been tested for their prospective use as a biocontrol agent of water hyacinth in Kerala (Praveena and Naseema, 2004). More recently mycoherbicidal potential of indigenous isolate of *Alternaria japonica* and *Phaeoacremonium italicum* has been evaluated for the control of water hyacinth (Dutta *et al*., 2015; Singh *et al*., 2016)

Thus the aim of this study was to explore the pathogenic mycobiota associated with *E. crassipes* and identify a suitable pathogen which could be developed into a mycoherbicide for possibly checking the spread and growth of water hyacinth in Harike wetland.

MATERIALS AND METHODS

Collection and isolation of the fungal pathogens

Infected plant samples (stolons, leaves and swollen bases) exhibiting necrotic spots and chlorotic symptoms were collected from the Harike wetland, Punjab during rainy and early winter seasons in the year 2012. These were stored in sterile polyethylene bags and transported to the laboratory, stored at 4ºC and processed within 24 h. Surface debris of the plant parts were removed by washing them under running tap water for 15 min. Subsequently they were swapped dry using sterile blotting paper. Leaf pieces were subsequently washed with 1% sodium hypochlorite for 1 to 2 min followed 30% ethanol for 1 min and then finally rinsed with sterile water thrice and air dried in laminar air flow. The infected portions were then dissected into 1×1 cm segments using a sterile blade. These surface sterilized plant segments were further cut into 2 to 4 mm segments and inoculated on Potato Dextrose Agar (PDA) medium (initial pH 4.5, HiMedia) supplemented with streptomycin (1mg/mL). Maximum of eight segments were inoculated per plate and incubated at 26±1°C for 7-days. The obtained fungal isolates were then sub-cultured over PDA plate as pure cultures and subsequently stored on a PDA slants containing 15% glycerol. These were tentatively identified using standard mycological keys given by Barnett and Hunter (1998).

Inoculum production

Fungal spores were harvested by flooding 10 d old culture of each isolate grown on water agar at 26 ± 2 ºC with sterile distilled water. Subsequently the spore concentration

was adjusted to 1 x 10^6 spores /mL using a hemocytometer and then mixed with 0.05% of Tween 20 (Sigma Aldrich).

In vitro detached leaf pathogenicity assay

Healthy *Eichhornia* leaves were sprayed with spore concentration of 1x10^6 spores/mL using a hand sprayer. Three leaves were used for per fungal isolate were used in the assay. These were then placed in an aseptic moist chamber prepared in 90 mm petri dishes and kept in a growth chamber with controlled conditions of 26 ± 2°C; 75 ± 15% relative humidity and 15 h (7350 lx) illumination for a period of one week. Leaves were visually examined every 24 h and percentage of symptomatic area of the leaves were recorded. All the tests were performed in triplicates (Saxena and Pandey, 2009). The disease progression during the *in vitro* detached leaf bioassay was established by determining the Area under Disease Progressive Curve (AUDPC) value of each isolate was calculated by the formula:

$$\text{AUDPC} = \sum_{i}^{n-1} [(y_i + y_{i+2})/2) (t_{i+1} - t_i)]$$

where

y_i is the assessment of the disease (percentage, proportion, ordinal score etc.) at the *i*th observation,

t_i is time (in days, hours etc.) at the *i*th observation, and

n is the total number of observations.

Whole plant bioassay for pathogenicity

The best isolate exhibiting highest pathogenicity in the în vitro detached leaf bioassay was taken up for further evaluation of pathogenicity on whole plants. Briefly 4 to 6 week old plants of *E. crassipes* were grown in tubs with illumination of 12 h daily for a period of one week for acclimatization under laboratory conditions. These were then sprayed with fungal inoculum of a spore concentration of 1x 10^6 spores/mL. Ten replicates were used in the experimental as well as in the control set. The control set were received only sterile distilled water (SDW). Plants were observed daily for disease severity (Saxena and Pandey, 2009). The experiment was repeated thrice.

Molecular identification of the selected fungus

The genomic DNA of the selected fungal isolate (#19BJSS) was extracted using Wizard® Genomic DNA purification kit (Promega, USA). For the DNA isolation, 2 to 3 mycelial discs (10 mm) of 4 to 5 d old selected fungal culture were scooped out and crushed to very fine powder in mortar and pestle by using liquid nitrogen. The powder was immediately transferred to the 2.0 mL micro centrifuge tube and the further extraction was carried out as per the instructions of the kit manufacturer. The internal transcribed spacer (ITS) regions of the nuclear rDNA was amplified using ITS1 (5' TCCGTA GGTGAACCTGCGG 3') and ITS 4 (5' TCCTCCGCTTATTGATATGC 3') (White *et al.,* 1990) using MyCycler™ (Bio-Rad). The amplification was carried out in 25µl reaction mixture containing 25ng of extracted genomic DNA, 0.8 µM of each primer (ITS1 and ITS4), 0.2mM of dNTP, 1.5 mM $MgCl_2$, 1.5U of Taq DNA Polymerase in 10 X Taq buffer. The Thermal cycling conditions consisted of initial denaturation at 96°C for 5 min followed by 39 cycles of 95°C for 45 s, 60°C for 45 s, 72°C for 45 s followed by final extension at 72°C for 5 min. The amplicons were purified by using the Wizard® SV Gel and PCR clean up system kit (Promega, USA). Gel imaging was performed under UV light in Bio-Rad Gel documentation System using Quantity-1-D analysis software.

Sequencing and Phylogenetic analysis

Purified products (amplicons) were sent for direct sequencing to Chromus Biotech, Bangalore. The Final sequence was obtained by assembling the obtained sequences using Sequencher ver.5 (www.genecodes.com). Sequences obtained in the present work were submitted in the NCBI database with Accession No. HG738871. The final sequence was subjected for BLAST similarity search against the database

Table 1. List of isolates and their accession number used in the phylogenetic analysis of #19BJSS (GenBank Accession HG 738871)

Name	Isolate number	Accession number*	Host	Place
Diplodia mutila	A24-AA-2	KP026317	*Amelanchir annifolia*	Canada
Diplodia mutila	1476	KM580526	*Vitis vinifera*	Chile
Diplodia mutila	4D33	KF778779	*Juglans regia*	USA
Diplodia mutila	KER-U-DMAPR1	KF535901	*Prunus armenica*	Iran
Diplodia mutila	BEI36	KT954166	*Vitis vinifera*	France
Diplodia seriata	DA221	KC960900	Pear	China
Diplodia seriata	KER-U-DSGG2	KC535906	*Prunus domestica*	Iran
Diplodia scrobiculata	CBS109944	DQ458899	*Pinus greggi*	Mexico
Diplodia mutila	BSDP1	KC789072	Date Palm	Iran
S. sapinea	CBS 393.84	DQ458895	*Pinus nigra*	Netherlands
S. sapinea	WA0000019144	JX981458	*Lycopodium sp.*	Poland
B. rhodina	CSS	GU226856	*Vitis vinifera*	China
B. rhodina	3-5	GU323603	-	China
B.eucalyptorum	MUCC387	DQ131571	*Eucalyptus sp.*	Australia
B.eucalypticola	MUCC388	DQ131571	*Eucalyptus globus*	Australia
A.niger	BGD22	HM107005	*Rhizophora stylosa*	China

maintained by NCBI to ascertain the homology with closely related organisms (Table 1). The sequences showing highest similarity (over 98%) for the locus were initially selected and included in the phylogenetic analysis and aligned with the respective sequence (HG738871) obtained in the present work by using ClustalW. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2004) and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 5). All positions containing gaps and missing data were eliminated. Clade stability was assessed by bootstrap analysis with 1000 bootstrap replicates.

RESULTS AND DISCUSSIONS

Isolation of Pathogenic fungi

The diseased leaves and stolons of *Eichhornia crassipes* collected from the Harike wetland possessed leaf spots, necrotic flecks, leaf blights and led to isolation of 30 isolates which comprised 11 genera (Table 2). The frequency of occurrence of *Alternaria* sp. is 26.7% followed by *Fusarium* with 20%, *Penicillium* and *Aspergillus* species with 13.3% and *Botryosphaeria* species with 6.7 %. The other fungi which were isolated during the sampling were *Acremonium* sp., *Nigrospora* sp., *Curvularia* sp., *Trichoderma* sp., *Didymella* sp. and *Artheminium*sp.

In vitro detached leaf pathogenicity assay

All the fungal isolates recovered from the diseased samples of *E. crassipes* were tentatively identified and tested using an *în vitro* detached leaf bioassay. Out of the 30 isolates tested, only *Diplodia* species #19BJSS exhibited 98% kill after 7 days post inoculation. The symptoms of the disease started appearing after 48 hpi and 60% kill was observed by 96 hpi. The symptoms include chlorosis of leaves as well as black necrotic spots leading to necrosis and death of the leaves by the end of the assay (Fig 1). The other isolates

Table 2. Fungal isolates inhabiting *Eichhornia crassipes* and related *în vitro* pathogenicity test on detached leaves

Culture code	Tentative identification	Leaf area damaged (%) after different hours post inoculation (hpi)					
		48	72	96	120	144	168
#1 BJSS	*Alternaria* sp.	10 ± 0^{d}	18 ± 2^{c}	23 ± 2^{bc}	30 ± 4^{b}	38 ± 2^{a}	43 ± 2^{a}
#2 BJSS	*Aspergillus* sp.	0^{c}	0^{c}	8 ± 2^{b}	10 ± 0^{ab}	12 ± 2^{ab}	13 ± 2^{a}
#3 BJSS	*Fusarium* sp.	0^{d}	10 ± 0^{c}	15 ± 0^{b}	22 ± 2^{a}	23 ± 2^{a}	25 ± 0^{a}
#4 BJSS	*Fusarium* sp.	0^{c}	5 ± 0^{bc}	12 ± 2^{b}	20 ± 4^{a}	23 ± 2^{a}	23 ± 2^{a}
#5 BJSS	*Alternaria* sp.	17 ± 2^{de}	32 ± 8^{cd}	50 ± 11^{bc}	70 ± 11^{ab}	82 ± 8^{a}	87 ± 2^{a}
#7 BJSS	*Botryosphaeria* sp.	20 ± 4^{d}	40 ± 4^{c}	61 ± 3^{b}	80 ± 0^{a}	83 ± 2^{a}	88 ± 2^{a}
#9 BJSS	*Fusarium* sp.	15 ± 2^{e}	28 ± 2^{d}	45 ± 4^{c}	55 ± 4^{b}	67 ± 2^{a}	73 ± 2^{a}
#10 BJSS	*Fusarium* sp.	35 ± 4^{e}	45 ± 0^{d}	55 ± 4^{c}	63 ± 2^{bc}	68 ± 2^{ab}	73 ± 2^{a}
#11 BJSS	*Acremonium* sp.	10 ± 4^{ef}	22 ± 8^{de}	37 ± 10^{cd}	48 ± 5^{bc}	62 ± 2^{ab}	68 ± 2^{a}
#12 BJSS	*Nigrospora* sp.	0^{c}	0^{c}	5 ± 0^{b}	5 ± 0^{b}	8 ± 2^{a}	10 ± 0^{a}
#14 BJSS	*Alternaria* sp.	22 ± 2^{e}	32 ± 2^{d}	43 ± 2^{c}	52 ± 2^{b}	63 ± 2^{a}	68 ± 2^{a}
#15 BJSS	*Penicillium* sp.	0^{d}	7 ± 2^{cd}	17 ± 2^{bc}	27 ± 6^{b}	40 ± 4^{a}	50 ± 4^{a}
#17 BJSS	*Penicillium* sp.	0^{f}	28 ± 2^{e}	42 ± 2^{d}	54 ± 3^{c}	63 ± 2^{b}	74 ± 3^{a}
#19 BJSS	*Diplodia* sp.	26 ± 3^{e}	40 ± 4^{d}	60 ± 4^{c}	82 ± 5^{b}	93 ± 6^{ab}	98 ± 2^{a}
#20 BJSS	*Alternaria* sp.	0^{e}	10 ± 4^{d}	20 ± 4^{c}	27 ± 2^{bc}	33 ± 2^{b}	43 ± 2^{a}
#21 BJSS	*Alternaria* sp.	0	0	0	0	0	0
#23 BJSS	*Aspergillus* sp.	0	0	0	0	0	0
#27 BJSS	*Fusarium* sp.	0	0	0	0	0	0
#29 BJSS	*Fusarium* sp.	0	0	0	0	0	0
#34 BJSS	*Curvularia* sp.	0	0	0	0	0	0
#38 BJSS	*Artheminium* sp.	0^{d}	0^{d}	0^{d}	13 ± 2^{c}	23 ± 2^{b}	32 ± 2^{a}
#42 BJSS	*Alternaria* sp.	0^{d}	0^{d}	0^{d}	5 ± 0^{c}	13 ± 2^{b}	22 ± 2^{a}
#44 BJSS	*Penicillium* sp.	0	0	0	5	5	10
#52 BJSS	*Didymella* sp.	0	0	0	0	0	0
#54 BJSS	*Trichoderma* sp.	0	0	0	0	0	0
#57 BJSS	*Alternaria* sp.	0^{d}	0^{d}	15 ± 0^{c}	20 ± 4^{c}	28 ± 2^{b}	37 ± 2^{a}
#14 BJSSS	*Aspergillus* sp.	0	0	0	0	0	0
#16 BJSSS	*Penicillium* sp.	0	0	0	0	0	0
#17 BJSSS	*Alternaria* sp.	0^{e}	0^{e}	5 ± 0^{d}	15 ± 0^{c}	22 ± 2^{b}	38 ± 2^{a}
#19 BJSSS	*Aspergillus* sp.	0	0	0	0	0	0

*Mean values along with their Standard error (±) are given in the table. Mean values represented by same alphabets in each row are not significantly different by Tukey's post hoc test at $p<0.05$

which also exhibited pathogenic potential were #7BJSS *Botryosphaeria* species followed by # 5 BJSS *Alternaria* species respectively with 88% and 87%, respectively (Table 2). Pathogenic potential of the different isolates tested were significantly different by one way ANOVA at $p<0.05$: $F (7, 232) =11.13$, $p\leq0.0001$. #19BJSS *Diplodia* species has not been reported as a pathogen or a biological

control agent of *Eichhornia crassipes*. Higher AUDPC of # 19BJSS also indicated that it possesses a higher pathogenic potential as compared to #7BJSS and # 5 BJSS (Fig.2). Hence #19BJSS *Diplodia* species was further selected to assess its potential as an innundative biological control using whole plant bioassay.

Fig.1. *în vitro* detached leaf assay of spores of #19BJSS (*Diplodia mutila*) 168 hpi.

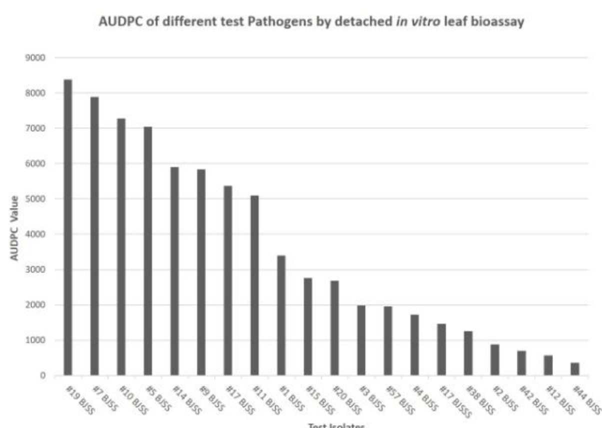

Fig.2. AUDPC of different test pathogens by *în vitro* detached leaf bioassay.

Whole plant bioassay for pathogenicity

In the whole plant bioassay the disease onset began after 2 d after inoculation. 50% damage of the whole plants was observed at the 4th day while above 90% death by 6thday after spore application (Figs.3, 4). The plants eventually died on the 7th day. There was not a significant difference in AUDPC observed in the *in vitro* leaf assay and whole plant bioassay with values of 8380 and 7842 respectively indicating its pathogenic potential.

Molecular identification of #19 BJSS

The Phylogenetic relationship of the isolate under study was carried out by sequencing the ITS region of the isolate. The sequence, thus obtained was showing 98% sequence similarity with *Sphaeropsis sp, Diplodia* sp. and *Botryosphaeriacae* sp. Hence, the present study undertaken the 17 sequences comprising *Diplodia mutila, Diplodia seriata*, *S. sapinea, B. rhodina* and *B. eucalyptorum.* The Neighbor-Joining tree was divided into three clades viz. Clade I, II and III. Clade I was further sectioned into two subclades- Sub-clade I which clustered isolate under study (#19BJSS) along with six strains of *Diplodia mutila* with high bootstrap support. Sub- clade II grouped strains of *D. seriata* and *S. sapinea.* However, *B. rhodina* and *B. eucalyptorum* formed their clades namely clade II and III respectively. *Aspergillus niger* was chosen as out-group to root the tree (Fig.5).

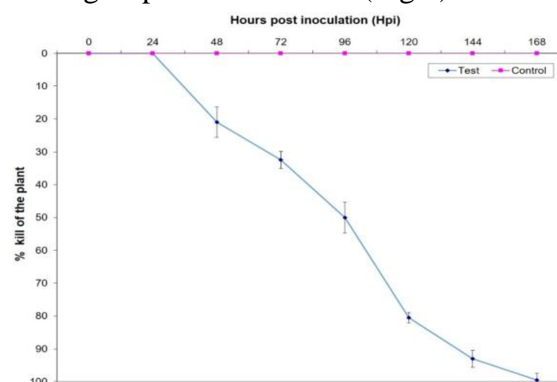

Fig.3. Disease progress caused by spores spray (1x10^6 spores/mL) of #19BJSS (*Diplodia mutila*) during whole plant bioassay.

Fig. 4. Whole plant bioassay exhibiting the kill caused by spore suspension (1 X 10^6 spores/ mL) of # 19BJSS (*Diplodia mutila*).

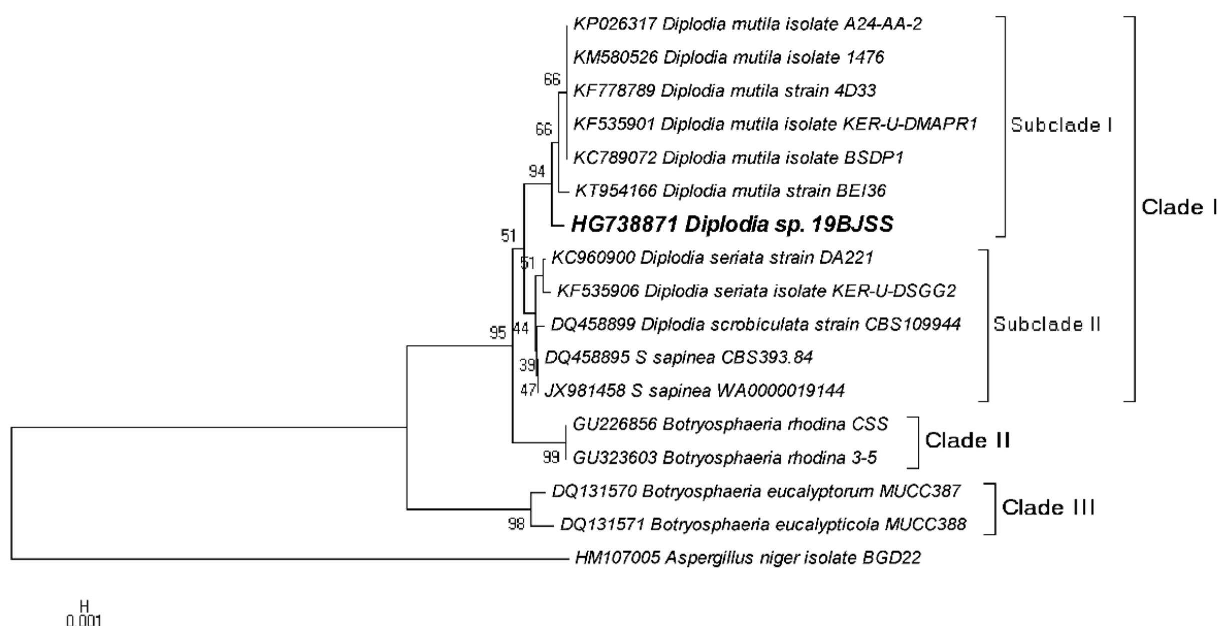

Fig. 5. Phylogenetic placement of #19 BJSS.

The Botryosphaeriaceae family encompasses a range of morphologically diverse fungi which exist has opportunistic pathogens, endophytes or saprobes which predominantly attack the woody hosts (Slippers and Wingfield, 2007). Botryosphaeriaceae comprises of anamorphic and teleomorphic states of *Lasiodiplodia*, *Diplodia*, *Botryosphaeria*, *Fusicoccum*, *Dothiorella* and *Sphaeropsis* species. *Diplodia* species have been found to be associated with different disease symptoms such as canker, gummosis, fruit rot, dieback and twig blight (Lazzizera e*t al.*, 2008; Phillips *et al.,* 2012; Abdollahzadeh *et al.,* 2013). Similarly some species *of Diplodia* namely *D. corticola*, *D. mutila*, *D. pinea* and *D. seriata* have been reported as well-known pathogens of woody plants (Phillips *et al.,* 2012). Recently *D. corticola* has been reported to cause stem cankers in *Quercus rubra* (Martin *et al.,* 2016); *D. mutila* causing branch cankers in bristlecone Fir in California (Sims *et al.,* 2016); *D. bulgarica* has been reported as a new pathogen on Apple trees in Iran and Bulgaria (Abdollahzadeh, 2015); *D. seriata* causing dieback and cankers in *Prunus laurocerasus* (Quaglia *et.al,* 2014). The diseases caused by *Diplodia* species have been associated with the onset of the stress factors and therefore it become imperative to isolate, identify and understand ecology and pathogenicity of fungi under diverse existence. More recently *Diplodia* species have been reported to exist as an endophyte in mangroves from South Africa (Osoria *et al.,* 2016). In the present publication we have identified *Diplodia mutila* as a pathogen for the very first time on aquatic weed *E. crassipes* which is non-woody in nature. ITS sequencing was primarily adopted to identify the pathogen which has been a method to report novel as well as known species of *Diplodia*. The pathogenicity of *D. mutila* was confirmed by establishing the Koch's postulates exhibiting characteristic symptoms of dieback. Further establishment of it safety on non–target plants and animals is essential for its possible use as a biological control agent for the management of water hyacinth.

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