Int.J.Curr.Microbiol.App.Sci (2018) *7*(2): 663-670

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 7 Number 02 (2018)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2018.702.082

Symptomatology and Molecular Characterization of Fungi Associated with Sigatoka Leaf Spot Disease of Banana in Kerala, India

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Keywords

Sigatoka leaf spot complex, Perithecia, ascospores, ITS – rDNA, Amplicons, *Mycosphaerella emusae*

Article Info

Accepted:
07 January 2018
Available Online:
10 February 2018

A B S T R A C T

Sigatoka leaf spot disease of banana is spreading at a faster rate causing a serious threat to banana cultivation in Kerala, the southern most state of Indian peninsula which enjoys a humid tropical climate. A survey was conducted under different agro-climatic zones prevailing in the state to study and document the symptomatology and etiology of the pathogen inciting Sigatoka leaf spot disease of banana occurring in the state of Kerala. The disease was noticed in most of the commercially grown varieties of banana *viz.*, Nendran (AAB), Grandnaine (AAA), Njalipoovan (AB) and Robusta (AAA) throughout the year, but become more severe with the onset of rains. The symptoms initially appeared as small light green to yellow dashes on the lower surface of the leaf which was visible only when the leaves was held against sunlight. This later changed into faint brown visible streaks. During the next stage, these streaks turned rusty brown on the adaxial surface of the leaves, which then developed into oval or elliptical brown spots with greyish centre surrounded by definite dark brown border with black pin head like fruiting bodies embedded in it. Upon heavy infection, the spots coalesced leading to complete necrosis of the leaves thereby destroying the functional green tissues of the leaves. This resulted in drastic reduction in the photosynthetic area causing an impact in yield reduction. The disease also resulted in premature ripening of the fruits affecting the fruit quality. The microscopic observations of these necrotic lesions revealed the presence of flask shaped telomorphic fruiting bodies called perithecia bearing asci and ascospores. The presence of the pathogen wasfurther confirmed by amplification of the ITS- rDNA region of the fungus using ITS 1 and ITS 4 primers which yielded amplicons of size ranging from 540-580 bp. The amplicons were further sequenced and blasted in NCBI which showed 97 - 100% sequence homology to *Mycosphaerella emusae.* Hence, the pathogen inciting Sigatoka leaf spot diseases on banana in Kerala was identified and confirmed as *Mycosphaerella emusae.*

Introduction

Banana is an important fruit crop grown in tropical countries. Though the crop is grown under diverse agroclimatic conditions, its cultivation is threatened by different diseases of which Sigatoka leaf spot disease caused by *Mycosphaerella* spp. is a serious constraint to

banana cultivation worldwide. In India, the this disease is more prevalent in states of Kerala, Tamil Nadu, Karnataka, Maharastra, Gujarat, West Bengal and Tripura where the maximum disease severity of90-100% was reported on different cultivars.

Globally, three species of *Mycosphaerella viz.*, *Mycosphaerella fijiensis*, *M. musicola* and *M. eumusae* have been reported to cause different Sigatoka leaf spot diseases like black sigatoka, yellow sigatoka and emusae leaf spot respectively (Arzanlou *et al.,* 2007).

The symptoms produced by all the three *Mycosphaerella* spp. on banana plants appeared to be very similar which complicates the identification of the pathogen based on visual symptoms (Surridge *et al.,* 2003).

Apart from this, the difficulty to culture the pathogen on artificial media limits the identification of the pathogen based on fungal structures. Therefore, it is essential to develop culture independent, specific molecular detection techniques for the identification of particular species of pathogen associated with Sigatoka leaf spot complex.

The reports on the rapid replacement of yellow Sigatoka disease by black Sigatoka in many tropical coastal regions (Jones, 2000) and the presence of emusae leaf spot caused by *Mycospaherella emusae* in South India (Carlier *et al.,* 2000b) necessitated the need to identify the pathogen associated with Sigatoka leaf spot disease in Kerala.

Therefore, in the present study a detailed survey was carried out in various agro-climatic zones of Kerala to understand the symptomatology and the particular pathogen associated with Sigatoka leaf spot disease complex occurring in banana especially in variety Nendran which is the most commercially growing variety of Kerala state.

Materials and Methods

Survey

A survey was conducted during 2016-2017 under different agro-climatic zones prevailing in the state *viz.*, Malapuram (Northern zone), Palakkad (Northern zone), Thrissur (Central zone), Ernakulam (Central zone), Wayanad (High range zone), Trivandrum (Southern zone) to study in detail the symptomatology, occurrence, severity and the pathogen associated with Sigatoka leaf spot disease complex. From an orchards of minimum 400 plants, 20 plants were randomly selected and scored to record the disease severity index and youngest spotted leaves. The leaves with different symptoms were collected from plants of different age groups and brought to the laboratory for further studies of the pathogen associated with the disease.

Morphology of the sexual stage of pathogen

The leaf sections having mature spots with stage 6 lesions were cut into $2cm^2$bits. These bits were dipped in 10% potassium hydroxide (which decolorizes plant tissue) (Udugama, 2002) overnight which was then washed 1-2 times in sterile water. The bits were then blot dried and the lesions were scrapped using a needle. The scraping were then transferred to a slide with a drop of lactophenol cotton blue stain and was observed under microscope for fungal structures.

Molecular characterization

Isolation of DNA

Molecular characterization of the pathogen associated with Sigatoka leaf spot disease of Kerala was carried out by isolating fungal DNA using *DNeasy Plant Mini Kit* from the lesions of the leaf samples (Surridge *et al.,* 2003) collected during the survey followed by

PCR amplification and sequencing of the ITS region of the isolated fungal DNA. About 100 mg of the leaf tissue containing mature lesions was homogenized using liquid nitrogen and the powdered tissue was transferred to a micro centrifuge tube. Four hundred µl of buffer AP1 was added and inverted for one minute. Four µl of RNAase A solution was added and vortexed vigourously for 30seconds. The homogenate was incubated at 65^{o}C in a water bath for 10 min. During incubation the homogenate was mixed 2 or 3 times by slowly inverting the tube. 130µl AP2 buffer was then added to the lysate, mixed well and incubated on ice for 5 min. The lysate was pipetted into the QIAshredder Minispin column placed in a 2ml collection tube and centrifuged at 14000 rpm for 2 min. The flow through liquid was collected into a new tube without disturbing the cell debris pellet. 1.5 volumes of AP3 buffer was added to the cleared lysate and was mixed by pipetting. Six hundred and fifty µl of the mixture was transferred into DNeasy Mini Spin column placed in a 2ml collection tube. The mixture was then centrifuged for one min at 8000 rpm and the flow through liquid was discarded. The column was then placed into 2ml collection tube, and 500µl of AW buffer was added, centrifuged at 8000 rpm for one min and the flow though liquid was discarded. Again 500µl of AW buffer was added to the column and centrifuged at 14000 rpm for 2 min to dry the membrane. The column was transferred to a new 1.5 ml tube and 100 µl of AE buffer was added directly into the DNeasy membrane and incubated at room temperature (15-25 ^{o} C) for 5 min. The column was then centrifuged at 8000 rpm for one min to elute the DNA. The eluted DNA was stored at 4^{o}C.

Agarose gel electrophoresis

The quality of the DNA isolated was checked using agarose gel electrophoresis. One µl of 6X gel-loading dye was added to 5µl of DNA and was mixed well by pipetting. The samples were loaded to 0.8% agarose gel prepared in 1X TAE containing 2µl ethidium bromide. Electrophoresis was performed with 1X TBE as electrophoresis buffer at 75 V until bromophenol dye front had migrated to the bottom of the gel. The gels were visualized in UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

PCR amplification and sequencing

PCR amplification was carried out by amplifying the ITS region of the isolated DNA using ITS 1 and ITS 4 primers. The PCR amplification reactions were carried out in 25 µl reaction volume which contained 12.5 µl of PCR master mix (Takara EmeraldAmp® GT PCR Master Mix), 0.5 µl ITS 1 primer, 0.5 µl of ITS 4 primer, 2 µl of DNA and 9.5 µlof distilled water. The PCR reaction conditions were standardized. The optimum reaction conditions were: initial denaturation of 94^{o}C for 3min followed by 35 cycles of denaturation at 94^{o}C for 45 sec, annealing at 60.4^{o}C for 45 sec, elongation at 72 ^{o} C for 1 min with final elongation at 72 ^{o} C for 10 min. Ten µl PCR product were loaded along with 100 bp ladder on 1.2% agarose gel prepared in 1X TAE containing 2µl ethidium bromide. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad). The amplicons were eluted using gel elution kit and the eluted amplified product was sent to Agri Genome Pvt Ltd. for sequencing.

Phylogenetic analysis

The sequence data set based on the ITS-rDNA region of the pathogen associated with Sigatoka leaf spot disease of banana and other reference sequences were retrieved from NCBI Genbank database (USA) using BLAST algorithm and were compared. Multiple

sequence alignment was done using Clustal Omega and phylogenetic tree was constructed in Mega6 using UPGMA tree construction method.

Results and Discussion

Survey

The details of the preliminary survey conducted at different agro-climatic zones of Kerala are presented in Table 1. The disease severity index was found more in Thiruvanathapuram district with a Disease severity index of 27.40. The disease was found to be more intense with the onset of rains. The disease symptoms on the commercially grown varieties of *viz.*, Nendran (AAB), Grandnaine (AAA), Njalipoovan (AB) and Robusta (AAA) were documented and observed for symptom development. The symptoms were expressed in six stages (Fig. 1) as described below. Disease symptoms observed initially were designated as stage 1 as yellow pigmented spots on lower surface of the leaves (Fig. 1.1). This is more visible only when the leaves are held against sunlight. These spots later changed into visible faint rusty streaks on the under surface of the leaves which is designated as stage 2 (Fig. 1.2). During the next stage of disease development *i.e.*, stage 3, the brown streaks became elongated and appeared on both abaxial and adaxial surface of the leaf (Fig. 1.3). The streaks then developed into elliptical spot surrounded by yellow halo represented as stage 4 (Fig. 1.4). At stage 5, the elliptical spots enlarges into lesions with greyish centers surrounded by definite dark brown borders containing black pin headed like fruiting body embedded in it (Fig. 1.5). When the infection density is high, these lesions coalesced causing complete necrosis of the leaf (stage 6), thereby reducing the photosynthetic area of the leaves which in turn reduced the yield of the crop (Fig. 1.6).

Morphological characters

The leaf samples treated with 10% potassium hydroxide when observed under microscope revealed the presence of perithecia like structure which were closely associated with the lesions.

The immature perithecia (Fig. 2.1) appeared to be round in shape whereas mature perithecia (Fig. 2.2) were flask shaped, 30- 40 µm wide and were ostiolated. The cracking of mature perithecia resulted in the release of asci (Fig. 2.3) bearing eight ascospores inside it. Each of the ascospores (Fig. 2.4) was 3 septated and was twined around each other. No asexual fungal structures were noticed.

Molecular characterization

DNA isolation and PCR amplification

The PCR amplification of rDNA-ITS region of the fungal DNA isolated from the lesions of the banana leaf samples using ITS 1 and ITS 4 primers yielded an amplicon of size 540- 580 bp (Table 2; Fig. 3). The sequence analysis of rDNA-ITS region using BLAST algorithm showed 97 - 100% sequence homology to *Mycosphaerella emusae* reported from banana plant.

Phylogenetic analysis

All the isolates inciting Sigatoka leaf spot disease were grouped into two major clusters A and B (Fig. 4). The major cluster A was divided into two sub clusters A1 and A2. The isolates *M. emusae*S6, *M. emusae*S7, *M. emusae*S2, *M. emusae*S4, *M. emusae*S12, *M. emusae*S5, *M. emusae*S11, *M. emusae*S10, *M. emusae*S8, *M. emusae*S1 were clustered together in sub cluster A1 showing close evolutionary origin and these isolates were closely associated with the isolate from Trichy (KC966893.1).

Table.1 Sigatoka leaf spot disease incidence in different agroclimatic zones of Kerala

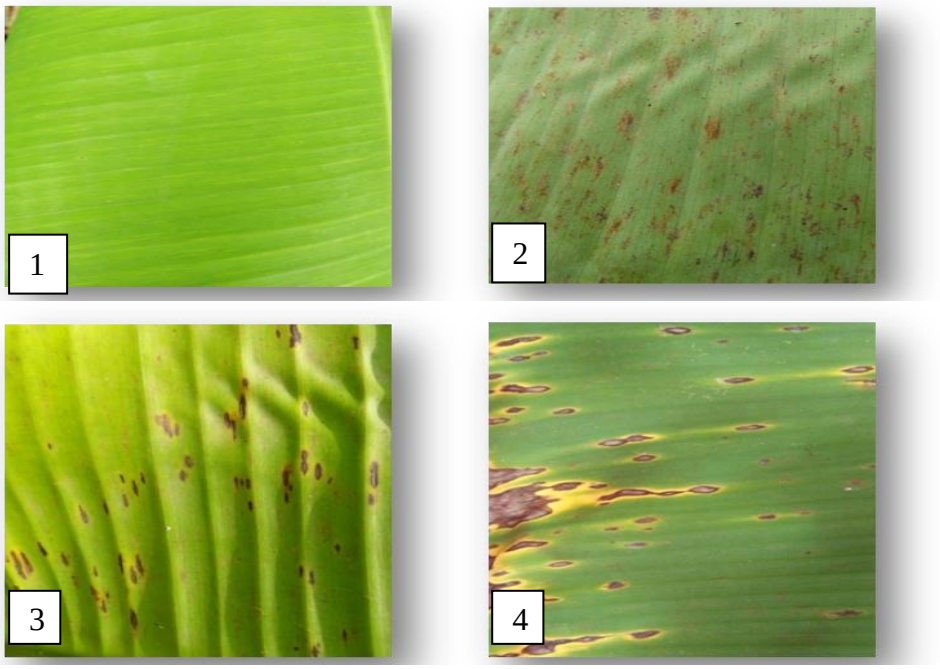
No:	District of survey	Stage of the crop surveyed (MAP)*	Variety	Youngest leaf spot (YLS)	Disease severity index (DSI)
S1.	Thrissur, Mellor	12	Swarnamuki	6	19.89
S2.	Thrissur, Madakkathara	7	Nendran	10	11.78
S3.	Palakkad, Pattambi	7	Nendran	9	12.50
S4.	Palakkad, Srikrishnapuram	5	Nendran	10	11.30
S5.	Malapuram, Vengara	10	Attunendran	8	16.78
S6.	Malapuram, SR Nagar	5	Nendran	10	13.48
S7.	Thiruvanathapuram,Peringamala	5	Chenkadali	6	27.40
S8.	Thiruvananthapuram,Neyyatinkkara	8	Nendran	11	21.79
S9.	Wayanad, Ambalavayil	9	Nendran	6	23.41
S10.	Wayanad, Sulthan Bethery	7	Nendran	7	19.08
S11.	Ernakulam, Pothanicad	7	Nendran	9	18.76
S12.	Ernakulam, Kalady	5	Nendran	10	14.84

MAP* - Months after planting

Table.2 Details of primers used

Target	Primer Name	Direction	Sequence (5' → 3')
ITS	ITS-1F	Forward	TCCGTAGGTGAACCTTGCGG
	ITS-4R	Reverse	TCCTCCGCTTATTGATATGC

Fig.1 Leaves showing different stages of Sigatoka leaf spot symptoms



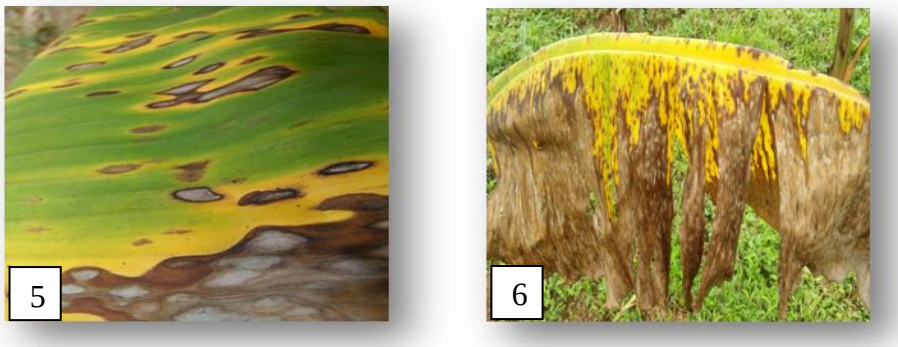


Fig.2 Sexual fruiting body of the pathogen

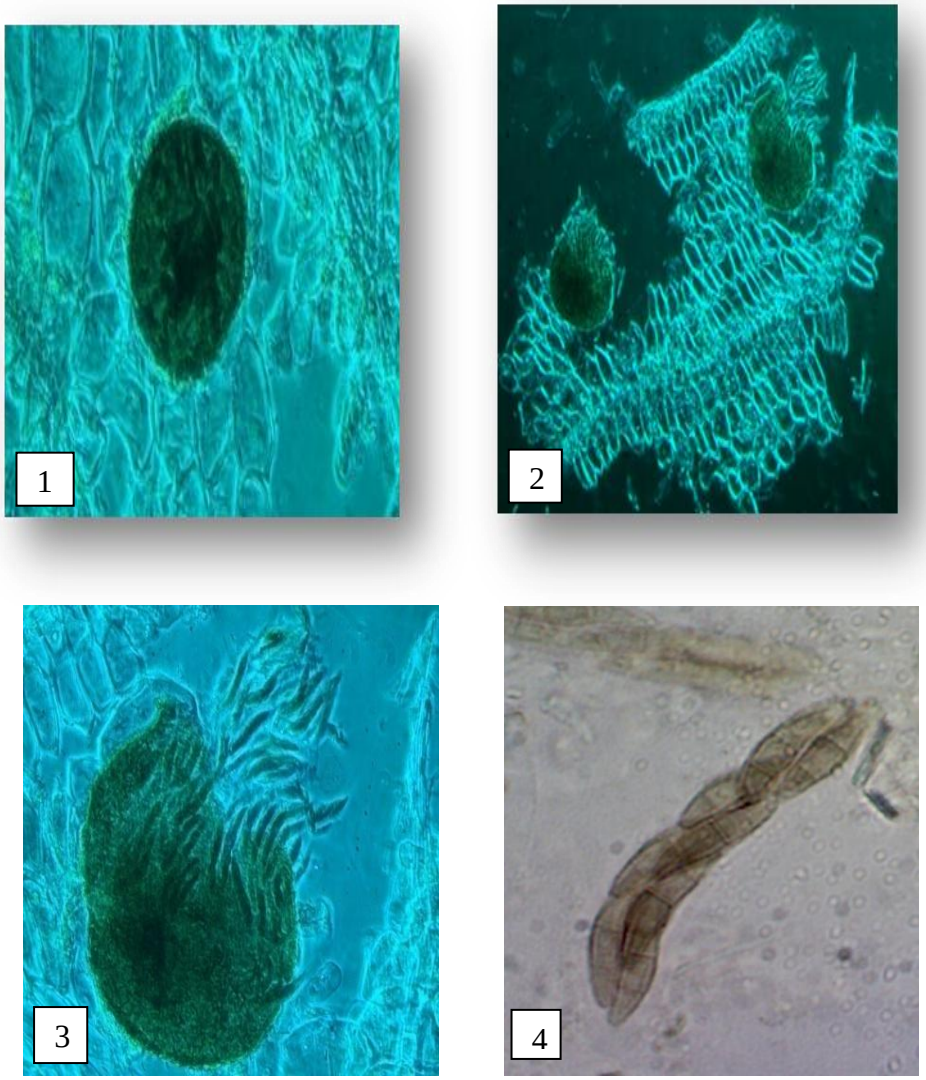


Fig.3 PCR amplicon obtained using ITS 1 and ITS 4 primers

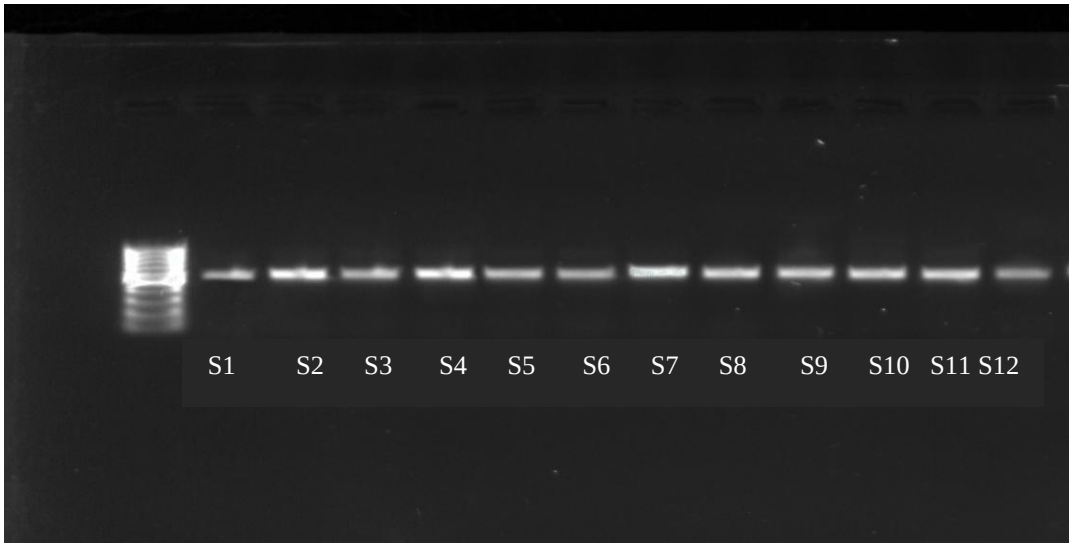
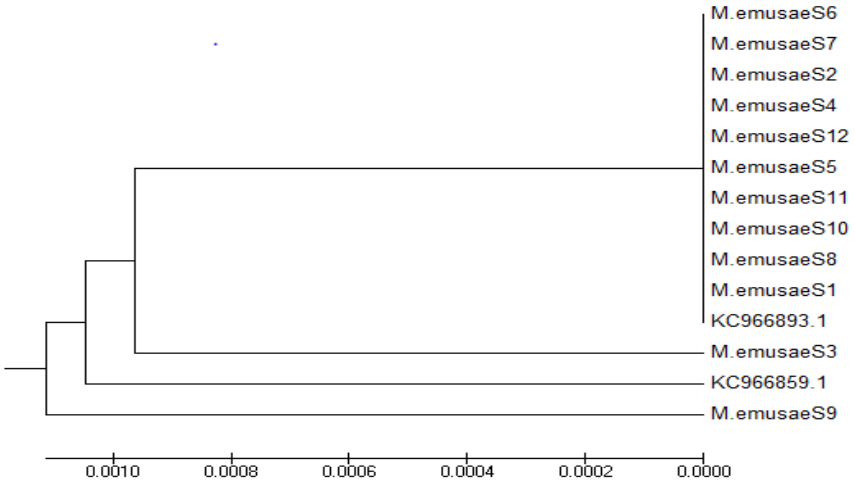


Fig.4 The evolutionary history was inferred using the UPGMA method. The evolutionary distances were computed using the Maximum Composite Likelihood method. Evolutionary analyses were conducted in MEGA6



The subcluster A2 contained only one isolate *M. emusae*S3 which showed close association to *M. emusae* (KC966859.1) reported from Trichy on banana. The isolate *M. emusae*S9 formed a single cluster B. Hence, the results of phylogenetic analysis indicated the presence of genetic diversity among *M. emusae* isolates collected from different agro-climatic zones of Kerala.

The three species of *Mycosphaerella viz., Mycosphaerella fijiensis*, *M. musicola* and *M. eumusae* inciting black Sigatoka, yellow Sigatoka and septoria leaf spot in banana are difficult to identify based on their symptoms and their cultural characters (Carlier *et al.*, 2000a). Therefore, the identification of the fungi associated with Sigatoka leaf spot of banana is very important for the effective management of leaf spot disease of banana, which otherwise result in higher yield reduction. Hence, the pathogen inciting Sigatoka leaf spot disease especially in banana variety Nendran grown in Kerala was

confirmed by PCR amplification of the rDNA- ITS region of the fungal DNA isolated from lesions of banana leaf collected during survey using ITS 1 and ITS 4 primers which yielded an amplicon of 540-580bp which was in accordance with the result of Devi and Thangavelu (2014). The analyses of the sequences of the amplicon using BLAST algorithm indicated 97-100% sequence homology to *Mycosphaerella emusae* reported from India on banana. Carlier *et al.*, (2000b) reported the causal agent of Sigatoka leaf spot disease in Southern India and Southeast Asia as *Mycosphaerella emusae* based on the sequences of ITS region and 5.8S ribosomal DNA regions of the pathogen. Jones (2002) also indicated that *M. emusae* affected the cultivars which were highly resistant to *M.fijiensis* and *M.musicola* and the incidence of *M. emusae* was more prevalent in Southeast Asia. Hence, this study clearly indicates that the fungi associated with Sigatoka leaf spot disease in commercially cultivated varieties of banana in the state of Kerala is *M. emusae.*

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How to cite this article:

Milsha George, K. Anita Cherian, S. Beena and Namitha, P.M. 2018. Symptomatology and Molecular Characterization of Fungi Associated with Sigatoka Leaf Spot Disease of Banana in Kerala, India. *Int.J.Curr.Microbiol.App.Sci.* 7(02): 663-670.
doi: https://doi.org/10.20546/ijcmas.2018.702.082