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# Molecular Characterization And Phylogenetic Analysis Of The Pathogen Lasiodiplodia Pseudotheobromae Causing Black Leaf Spot Disease On Croton Bonplandianum (L), Plant.

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***Abstract: Lasiodiplodia pseudotheobromae is an important weed pathogen of Croton bonplandianum in the red gram crop fields. The aim of the present study was to molecular characterization and phylogenetic analysis of the fungal pathogen Lasiodiplodia pseudotheobromae .In this we will demonstrate that the DNA barcoding is the major tool for molecular analysis and Lasiodiplodia pseudotheobromae is represented by phylogentic tree for provides the systematic frame work for future analysis. The best phylogeny of the pathogen was given with the help of maximum parsimony (MP), maximum likelihood (ML), and neighbor joining (NJ) methods. As for the best of my knowledge the Lasiodiplodia pseudotheobromae the first reported in Croton bonplandium weed plant in Vinukondal mandal of Guntur district***

***Key words: Phylogeny, pathogen, rRNA sequences, Maximum parsimony (MP), Maximum likelihood (ML), Neighbour joining (NJ).***

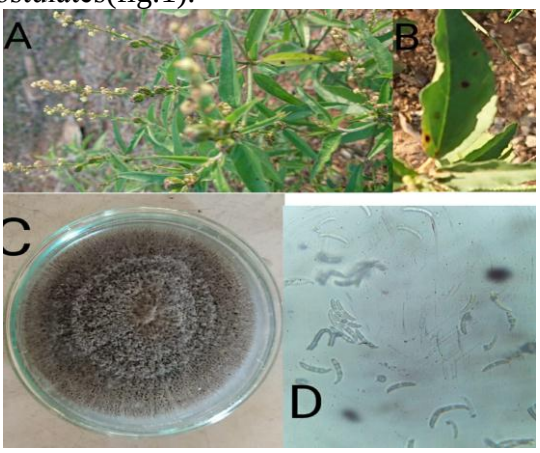
## 1. INTRODUCTION

The fungal pathogen *Lasiodiplodia pseudotheobromae* was a Bortyspaeriace family member, in Ascomycota. There are 21 species in *Lasiodiplodia* genus. The fungal family Botryspaeriaceae distributed world widely, and they have shown a great plant-host relationships includes pathogenic, saprophytic and entophytic relationship on Gymnosperms and Angiosperms. The Botryspaeriaceae family is an important pathogenic family in ascomycota.Botryspaeriaceae are morphologically diverse and characterized by multi to unilocular ascomata with multi layered walls.*Lasiodiplodia pesudotheobromae* was widely distributed in tropical and sub tropical regions. (Marques et al., 2013&Punithalingam, 1980).The main characteristics that distinguish. The fungus lives in endophytically and causes the diseases to the host plant. The pathogenic spores are spread by water or rain, and they also transmitted through infected plant materials and seeds (Punithalingam, 1976).It can also spread by insects, birds or soil.

For identification of fungi to a species level is a most important fundamental to many researchers in life sciences. It's true in applied and basic research fields. In both natural and agricultural fields, it's most important to identify correct plant pathogens, for identify the plant diseases (McNeil et al., 2004; &Wingfield et al., 2001). Furthermore, reliable species identification plays a central role in all studies relating to conservation biology and ecology, because all biological aspects of any given individual in an ecosystem can only be attributed meaningfully via an unambiguous identifier like a species name (Whittaker, 1970**)**.The molecular date is now used widely in fungal systematic and used for phylogeny identification, for valid description of a species. Therefore, molecular tools were readily embraced by the mycological community when they became available. This is exemplified by the fact that nearly 6,000 fungal sequences were ready to be published when the US-based National Institute of Health initiated GenBank in 1993, and the yearly sequence submissions increased rapidly to a total number of more than 2.4 million fungal sequences(Dominik et al., 2010).

## 2. MATERIALS & METHODS

**Isolation and cultures of the pathogen**: A fungal stain of *Lasiodiplodia pseudotheobromae* was isolated from the diseased spots of the leaf of the weed plant, *Croton bonplandium,* from the agricultural fields at Vinukonda mandal of Guntur district. The infected leaves were collected from the study area and washed thoroughly by running tap water to remove the unwanted dust or soil from the leaves. The infected portions of the leaves were cut into 1.0 to 1.5 cm small fragments with diseased spot. The leaf bits were surface sterilized by 70% alcohol by 3 times for 2 minutes and then reside with distilled water by 3 times. Then after the leaf bits were rinsed in 0.01% mercuric chloride solution ($Hgcl_2$) for 1 minute followed by washing with sterile autoclaved double distilled water for 3 times. The surface sterilized leaf bits were then transferred into PDA (Potato dextrose agar) Petri plates with 1% of streptomycin sulphate (antibiotic). These Petri plates were kept incubated at 25 ± 2°C for 2-3 days at 8 hrs of photoperiod in incubation chamberand confirmation of the pathogen was done according to the Koch's postulates(fig:1).




**Figure**

**1. Diseased plant from study area 2. Diseased leaf**

**3. Cultured petriplate 4. Spores of pathogen**

The growing mycelium in Petri plates were then transfers to slands for further study. The spore morphology was studied by using binocular microscope in Department of Botany Andhra University Visakhapatnam

**Pathogenicity testing**

The healthy weed plant of *Croton bonplandium* were maintained for the pathogenicity test of

the pathogen. The mycelium from the cultured pathogen after the spore formation about 5-7 days old was taken from the PDA cultures of the *Lasiodiplodia pseudotheobromae* isolate, to perform the pathogenicity tests by inoculation on healthy leaves of the tested plants. In this pathogenecity test we used the different concentrations of the spore suspensions**50**, **25**, **12**and **6** spores/microscope field areas by suing serial dilution technique with sterilized distilled water. The prepared spore suspensions were spray on the leaves of the tested plants and were kept in moist chamber for two days. Control plant without spore suspension was also maintained. After two days they were transferred from moist chamber to normal environmental conditions. The disease symptoms of leaf spots were observed after 5-7 days of spore sparing. The leaves were collected after 7-10 days, for the pathogen conformation.

After 10 days, the tested plants in the green house show the disease .The pathogen was reisolated from the infected leaves of the plants. In all the spore suspensions, 50spores/microscope field area shows the maximum disease intensity compare to others. After the 3-5 days of inoculation the pathogen shows the fully developed disease spots. All the inoculated plants in the green house show the pathogenecity of the same pathogen.

## 3. MOLECULAR ANALYSIS

For the molecular analysis of the pathogen, the disc of the growing fungal mycelium about 3-5 days on PDA(Potato dextrose agar) was taken to inoculate in 20 ml of PDB(potato dextrose broth), and the culture were placed in an incubation chamber for about 5(five) days. After 5 days of the incubation, the mycelium shows fully developed mycelium, this mycelium was recovering by using muslin cloth with distilled water. This mycelium was used for the extracting the genomic DNA.

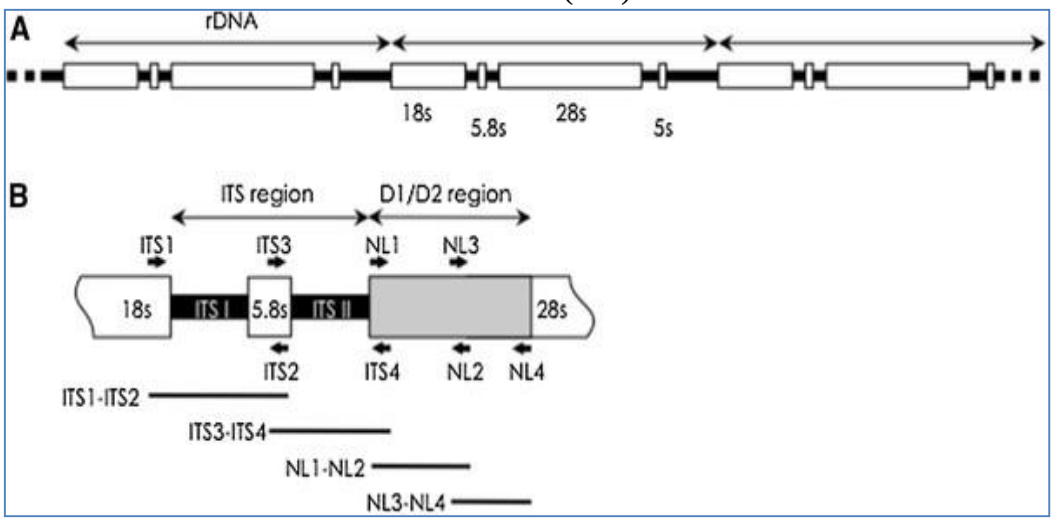
50-10mg (wet weight) fungal cells that have suspended in up to200 ul of water or isolotonic buffer( e.g. PBS) to a ZR Bashing Bead™ Lysis tube. Add 750 ul lysis solutions to the tube. Secured tubes in a bead beater fitted with a 2 ml tube holder assembly (e.g., Disruptor Genie) and process at maximum speed for 5 minutes. Centrifuged the ZR Bashing Bead™ Lysis Tube in a micro centrifuge at 10,000 x g for 1 minute.Transferred up to 400 µl supernatant to a Zymo-Spin™ IV Spin Filter (orange top) in a Collection Tube and centrifuge at 7,000 rpm (~7,000 x g) for 1 minute. Snap off the base of the Zymo-Spin IV™ Spin Filter prior to use.Added 1,200 µl of Fungal DNA Binding Buffer to the filtrate in the Collection Tube from Step. Transferred 800 µl of the mixture from Step 5 to a Zymo-Spin™ IIC Column in a Collection Tube and centrifuge at 10,000 x g for 1 minute. Discarded the flow through from the Collection Tube and repeat Step .Added 200µl DNA Pre-Wash Buffer to the Zymo-Spin™ IIC Column in a new Collection Tube and centrifuge at 10,000 x g for 1 minute. Added 500µl Fungal DNA Wash Buffer to the Zymo-Spin™ IIC Column and centrifuge at 10,000 x g for 1 minute.10. Transferred the Zymo-Spin™ IIC Column to a clean

1.5 ml micro centrifuge tube and add 100µl (25µl minimum) DNA Elution Buffer directly to the column matrix. Centrifuge at 10,000 x g for 30 seconds to elute the DNA. The concentration and purity of the DNA was checked by Thermo Scientific Nanodrop Spectrometer and on 1% agarose gel.

The polymerase chain reaction mixture about (25ul) contains - 10 x polymerase mixture2.5 µl, 10Mm $Mgcl_2$ 1.5 mM, 10 Mm dNTP, taq polymerase0.3µl, F-27 Primer/ITS5F (10 pmol/ul) 1.0µl, R-1492 primer/NL4R (10 pmol/ul)1.0µl, Template (10-25 ng) 1.0µl, Sterile $ddH_2O$.

The PCR reaction procedure had the following steps: (1) Initial denaturation at 95 °C for 5 min; (2) Denaturation at 95 °C for 30 s, (3) Annealing at 55 °C for 30 s and extending at 72 °C for 33 cycles; and (4) Extending at 72 °C for 10 min.

The classical sets of primers were selected to amplify the target gene they are ITS-1 And ITS-4 (ITS).




ITS5F –5'GGAAGTAAAAGTCGTAACAAGG 3'

The DNA amplification and sequencing were carried out with using a program according ZR fungal DNA MINI PRE.The PCR products were sequenced by MTCC gene bank Chandigarh to avoid errors in sequencing, DNA strands were spliced using forward and reverse primers.

**Phylogenetic analysis**

The resulting ITS gene sequencing was BLASTED, other sequences needed for phylogenetic analysis were obtained from the gene bank data base of NCBI. The foreword and reversed ITS sequences were assumed and aligned by molecular evolutionary genetic analysis by MEGA softwere. The phylogenetic tree was constructed by using the -Joining (NJ).which was validated using bootstrap analyses with 1000 repetitions and, The phylogenetic tree was contrastued on the concatenated sequences of ITS.

## 4. RESULTS

**Isolation and morphology of fungal pathogen**

The sample collected from the symptomatic weed plant *Croton bonplandiaum.L* shows the disease, the causing pathogen was *Lasiodiplodia pseudothobromae*.At the starting of the disease, the leaves shows the brown to black colour and round or irregular shape, but later it becomes dark bown to black and irregular in shape. The cultured Petri plate shows the white mycelium with black spores, and spores were formed in5 - 7 days old culture. The Isolatiolated pathogen was identified by MTCC gene Bank Chandigarh (Fig: 2) and the identified pathogen was *Lasiodiplodia pseudotheobromae.* Pathogen shows the black spores with white mycelium and hyaline, aseptate and septate conidia with longitudinal striation.

सूक्ष्मजीव प्ररूप संवर्धन संग्रह एवं जीन बैंक
MICROBIAL TYPE CULTURE COLLECTION & GENE BANK
सूक्ष्मजीव प्रौद्योगिकी संस्थान
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18th Feb 2020

Dear Ms. Bikki ,

Please find enclosed herewith the result of ITS region sequencing on your fungi strains. Based on sequence analysis the strain was showing similarity as follows

| S.N. | Sample number | Identity | Percentage similarity (%) |
|---|---|---|---|
| 1. | Sample II | *Lasiodiplodia pseudotheobromae* | 100% |

A detailed list of sequencing report is annexed for your kind information.

Thank you for your interest in MTCC

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Scanned with CamScanner

Fig: 2 Identification of pathogen by MTCC gene bank

**Molecular and phylogeny analysis result**

The internal transcribed region (ITS) is the most frequently sequenced genetically marker of the fungi and it is routinely used to address the no. of questions from researchers like systematic , phylogeny , identification of stains and specimens at above and below the species level(Dominik et al., 2010).

The sequence of the ITS region of the cultured isolate *Lasiodiplodia pseudotheobromae* were successfully amplified by using the primers of ITS region of 16SrRNA sequence 5'GGAAGTAAAAGTCGTAACAAGG 3 respectively. The amplified ITS region shows the size of 500-600 bp respectively. For analysing the gene sequence, the sequences of other species of *Lasiodiplodia* were taken from the NCBI gene bank. After analysis of tested pathogen is the *Lasiodiplodia pseudotheobromae*, there were five isolated of *Lasiodiplodia pseudotheobromae* in one isolate *Lasiodiplodia pseudotheobromae* isolate A738 with 87% of similarity–and four Isolates CMW40982, SSB2B, Trs58, Trs70, NL8E1 with 100% similarity were identified .The four isolates of *Lasiodiplodia theobromae* FH14K03 , Lt1, B3179, B3185 were with 39% of similarity. And *Lasiodiplodia iranensis*two isolates MTZ45, MTU07 were shows 67% similarity and one isolate115 show 39% similarity with tested pathogen. The *Lasiodiplodia* species isolates La2 and La3 shows the 39% of similarity with the tested pathogen. The ITS regions of the tested pathogen conformed that *Lasiodiplodia pseudotheobromae* according to their molecular sequence of the ITS region of the 16SrRNA.

>GATCCGAGGTCACCTTGAGAAAAGTTCAGAAGGTTCGTCCGGCGGGCGACGCC
AACCACTCCAAAGCGAGGTGTATTCTACTACGCTTGAGGGCTGAACAGCCACCG
CCGAGGTCTTTGAGGCGCGTCCGCAGTGAGGACGGTGCCCAATTCCAAGCAGAG
CTTGAGGGTTGTAATGACGCTCGAACAGGCATGCCCCCCGGAATACCAAGGGGC
GCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTT
ATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAA
AGTTTTAGTTTATTAACTTGTTTATCAGACGTCTGCGTTTACTGACTGGAGTTTGG
AGGTCCTTTGGCGG <

ITS sequence of the ***Lasiodiplodia pseudotheobromae*** was mentioned in the (Fig: 3) IST5F + ITS4R region sequence of the pathogen *Lasiodiplodia pseudotheobromae* given by MTCC gene bank**.**

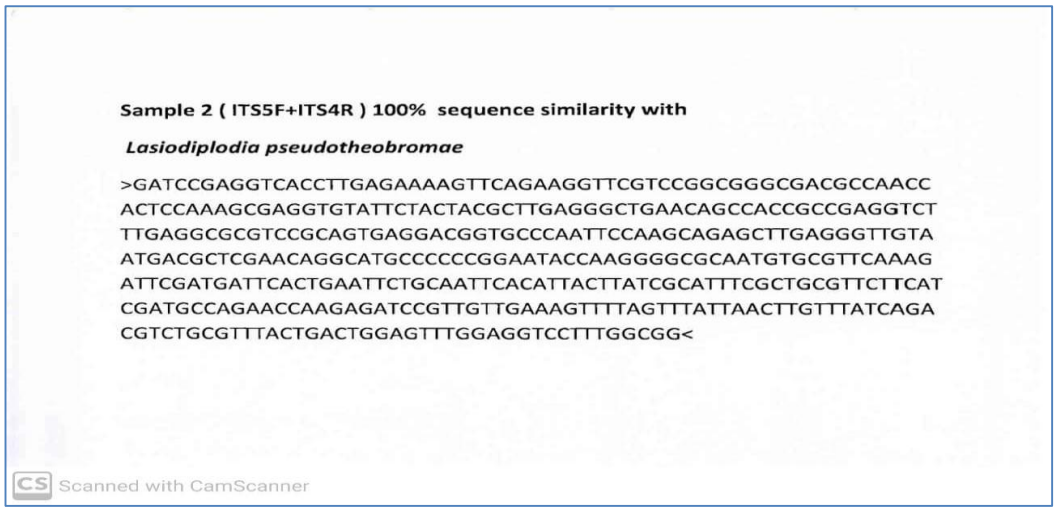


**Figure: 3**

ITS5F and ITS4R region sequence of the pathogen *Lasiodiplodia pseudotheobromae.*

To acquire phylogenetic analysis of the fungal pathogen *Lasiodiplodia pseudotheobromae*, the DNA sequence of the Inter transcribed unit (ITS) region of 16SrRNA was amplified sequenced. Phylogenetic tree is commonly defined as a leaf –labeled tree and that represents the evolutionary history of a set of taxa, possibly with branch lengths either rooted or unrooted. The phylogenetic tree were constructed by using the MEGA software and aligment due to sequence of the ITS regions of the tested pathogen. In early 1990sMEGA software functionally has evolved to include the creation and exploration of sequence alignments. The testedisolated were sequenced with the other species of the Lasiodiplodia isolates and they were analysis and sequenced taken from NCBI gene bank. In the results clearly shows that the tested conformed pathogen were *Lasiodiplodia pseudotheobromae* and it's clearly divided into Cades and groups and they were coming from common ancestor (Fig:4).In these we explained to define a phylogenetic network as any network in its represents the evolutionary relationships of the tested pathogen by nodes and edges.

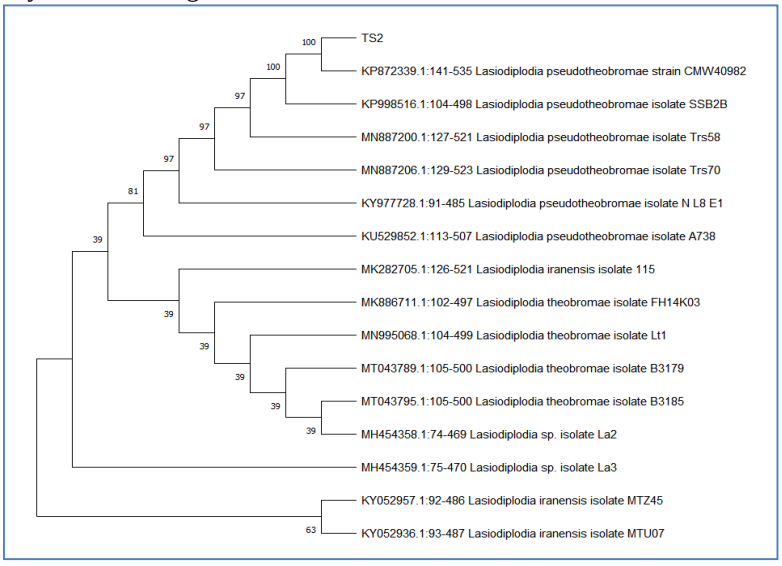


**Figure: 4**

**phylogenetic tree based on the ITS region of 16Sr RNA gene sequence of pathogen**

*Lasiodiplodia pseudotheobromae* with related species of the Lasiodiplodia isolated from the weed plant *Croton bonplandium*. The bootstrap values 1000 replicates are next to branches.

## 5. DISCUSSION

In the present study we identified the pathogen *Lasiodiplodia pseudotheobromae* associated with the host weed plant of the *Croton bonplandiaum*.L in the red gram agricultural crop fields at vinukonda mandal, Guntur District, Andhra Pradesh.

The *Lasiodiplodia pseudotheobromae* is important in agriculture and forestry as it causes cankers, stem-end rot, dieback and fruit rot (Ismail et al., 2012; Marques et al.,2013; & Sakalidis et al.,2011).Six species of *Lasiodiplodia* were associated with a variety of symptoms on a range of woody hosts in of these (*L. citricola, L. gilanensis, L. hormozganensis* and *L. iraniensis*) are recognised as new. All four species can be distinguished morphologically and phylogenetically from one another and from previously described species (Abdollahzadeh et all., 2010). Two *Lasiodiplodia* species, *L. pseudotheobromae*and *L. theobromae*, were identified and latter species is a widely distributed plant pathogen that has most commonly been reported from the tropics and subtropics (Punithalingam, 1980). *Lasiodiplodia pseudotheobromae* and *L.parva* are recognized as two new species in the *genusLasiodiplodia*, closely related to *L.theobromae*, Both species possess morphological features typical of the genus, namely slowly maturing conidia with thick walls and longitudinal striations resulting from melanin deposition on the inner surface of the wall (Punithalingam, 1976; & Punithalingam, 1980). Chinese hackberry stems canker disease caused by *L. Pseudotheobromae* in China (Liang et all.2019).

Worldwide, this species has a wide range. *L. Pseudotheobromae* is an aggressive species in Australia, Cameroon, Egypt, Brazil, Mexico, China and other countries (Begoude et al., 2010; Coutinho et al., 2011; Ismail et al., 2012; Maricamen et al.,2013 & Sakalidis et al.,2011).Although they are closely related, the size and shape of *L.pseudotheobromae* conidia differ from those of *L. Theobromae* which are larger and have orbicular ends (Alves et al., 2008). In addition, *L. pseudotheobromae* can also grow at 10°C and form deep-pink colonies at 35°C but *L. Theobromae* does not **(**Marques et al., 2013; &Netto et al., 2014).Species in *Lasiodiplodia* have been distinguished based on their DNA phylogeny in association with conidial morphology and size of paraphyses to differentiate *Lasiodiplodia species* including *L. crassispora, L. gonubiensis, L. rubropurpurea, L. theobromae and L. venezuelensis*. The pycnidia or fruiting body of the fungus is produced near the canker before spreading to neighboring hosts. Pathogens commonly enter the plant through wounds left by pruning of twigs and this may be the main reason for the spread of the disease. In addition,(Zhao et al.,2010**)** recently reported *L. pseudotheobromae* on *Mangifera sylvatica* and on other tropical and subtropical trees in China. This study represents the first report of *L.*

*pseudotheobromae* on mango in Egypt associated with severe twig and branch dieback, leading to tree mortality. *L. theobromae*, L. *pseudotheobromae* and the newly described *L. egyptiacae.* The latter new species is distinguished from other species of *Lasiodiplodia* based on morphological characters and phylogenetic inference. Several authors have in the past relied on DNA sequence data (ITS and TEF1-α) and morphological characteristics to separate species in this genus, namely conidia (shape, dimensions and septation), paraphyses size and septation) culture morphology and temperature requirements for growth (Abdollahzadeh et al., 2010; Alves et al., 2008; Burgess et al., 2006 ; & Damm et al.,2007).Although morphological characters can overlap (Abdollahzadeh et al., 2010 ; Charles, 1970 ;Kim et al.,2005 ; Pennycook &Samuels 1985 ; & Slippers et al., 2004a).They are still useful complimentary features when combined with DNA phylogeny to distinguish new species in the Botryosphaeriaceae. In the present study, the shape and length of paraphyses were used to differentiate *L. egyptiacae* from the phylogenetically closely related species such as*L. hormozganensis, L. parva and L. citricola*. (Burgess et al., 2006). relied on the septation of paraphyses to discriminate between *Lasiodiplodia spp*. and indicated that *L. gonubiensis, L. venezuelensis* and *L. Crassispora* have septate paraphyses, whereas in other species they were a-septate.

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