# PATHOGENICITY AND TOXIN PRODUCTION OF *CORYNESPORA CASSIICOLA* ISOLATES CAUSING CORYNESPORA LEAF FALL DISEASE IN *HEVEA BRASILIENSIS*

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*Corynespora cassiicola* is an important plant pathogenic *Ascomycete* causing Corynespora leaf fall (CLF) disease in *Hevea brasiliensis*. Phytotoxins produced by pathogens are the key factors in the development of a number of destructive diseases of crop plants. A small glycoprotein named cassiicolin was previously described as an important effector of *C. cassiicola.* The degree of aggressiveness and pathogenicity of seven *C. cassiicola* isolates were attempted in the present study. Aggressiveness of the isolates was studied by two different methods *viz*. the leaf wilt bioassay using crude toxin and spore inoculation in RRII 105 (susceptible clone) and Fx 516 (tolerant clone). *Cas* gene responsible for pathogenicity was detected in the isolates. Variability in the degree of virulence was observed among the *C. cassiicola* isolates. The isolates with no *Cas* gene could nevertheless generate moderate symptoms, suggesting the existence of other yet uncharacterized effectors.

**Key words:** *Cas* gene, Cassiicolin, *Corynespora cassiicola,* Corynespora leaf fall disease, *Hevea brasiliensis*, Pathogenicity test

## INTRODUCTION

Commercially cultivated clones of *Hevea brasiliensis* represent a very narrow genetic base possibly originating from few selected seedlings collected by Henry Wickham in 1876. *H. brasiliensis* being an economic crop its healthy existence is significant to its productivity output (Narayanan and Mydin, 2011). Rubber cultivation is under a constant threat of attack by native as well as exotic pathogenic fungal diseases due to genetic vulnerability of the *Hevea* clones. In rubber tree, *Corynespora cassiicola* causes the damaging Corynespora leaf fall (CLF) disease that affects rubber in all Asian and African countries (Chee, 1990). Corynespora leaf disease was first detected in India in 1958 in seedling nurseries (Ramakrishnan and Pillay, 1961), although association of the fungus with rubber leaves was reported from Sierra Leone (Deighton, 1936). Corynespora leaf disease in mature trees was reported in India from the plantations of South Karnataka (Rajalakshmi and Kothandarman, 1996). Corynespora disease is more severe during the refoliation period of rubber trees (December to April). High

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temperature and humidity during refoliation period were found to favor the disease incidence.

The symptoms observed in the nurseries are usually circular, rarely irregular amphigynous lesions on leaf lamina (1 to 8 mm in diameter). The disease spreads on the leaf veins, turning them dark brown. When veins and veinlets are affected, they appear like railway track markings as seen on geographical maps. The leaf tissues surrounding infected veins turn yellow and later to brown, and then the leaf falls off. The symptom is also described as 'fish bone' due to the similarity to the bones of small fishes. This symptom is due to the production of toxin at the infection locus (Liyanage and Liyanage, 1986, Joseph, 2004).

Phytotoxins produced by pathogens are the key factors in the development of a number of destructive diseases of crop plants. *C. cassiicola* pathogenicity in rubber tree is mediated by a small phytotoxic protein named cassiicolin which was first purified from the culture filtrate of a pathogenic isolate (CCP) from a rubber plantation in Philippines (Breton *et al.*, 2000; de Lamotte *et al*., 2007; Deon *et al*., 2012a) Cassiicolin is a 27 amino acid glycosylated protein, matured from a precursor protein (Cas1) endowed with a signal peptide that is involved in secretion (Deon *et al.*, 2012a) with an isoelectric point near to 3.18. The objective of the present study was to isolate the toxin from seven different *C. cassiicola* isolates and to study the aggressiveness of the isolates by *in-vitro* screening of the clones (Fx 516 and RRII 105), PCR amplification and detection of *Cas1* gene from the genomic DNA of the isolates.

## MATERIALS AND METHODS

### *Corynispora cassiicola* isolates

The study was carried out using seven isolates of *C. cassiicola* from the culture collection of Rubber Research Institute of India. The isolates were purified by single spore isolation and were multiplied on potato dextrose agar (PDA) medium. Mycelia disc (7 mm) taken from area near the edge of seven day old culture on potato dextrose agar (PDA) was inoculated centrally on petridish containing PDA. The plates were incubated at 25 - 28 °C with 12h alternations of light and dark for 10 days to obtain maximum growth and sporulation. Colony morphology such as colony color, colony texture and colony diameter was recorded.

### Crude toxin isolation

Four mycelial plugs each of 7 mm diameter obtained from 10 day old cultures of each isolates (cultured on PDA plates) were inoculated in 100 ml of modified Czapek Dox broth (CDB) in 250 ml Erlenmeyer flasks. Inoculated flasks were incubated without agitation for 12 days at room temperature with a photoperiod of 12 h light/12 h dark, filtered through Whatman No. 1 filter paper to separate the mycelia from the culture filtrate, lyophilized and stored at -80 °C. The leaf wilt assay (Breton *et al*., 2000) was used to determine the toxic activity of the filtrates.

Pathogenicity tests were conducted in two clones *viz*. RRII 105 (susceptible) and Fx 516 (tolerant) to detect the aggressiveness of isolates. Aggressiveness/pathogenicity was studied by leaf wilt bio assay and spore inoculation method.

In the leaf wilt bioassay, young leaflets of light green stage were excised under water from the petioles and immediately transferred to glass tubes containing five ml toxic fractions (1ml crude toxin diluted in 4ml sterilized water). Toxin free medium or distilled water served as control. The leaflets were incubated at 25 °C and 12 h photoperiod

and wilting intensity was assessed after at 48 hours

In the spore inoculation method, the conidia were collected and resuspended in sterile water. In total, 10 drops of conidial suspension (20 µl) were applied to the abaxial surface of detached young leaf lets at light green stage (Halle and Martin 1968). The leaflets were maintained in a moist chamber 25 °C for 72 h. Toxic activity is estimated by measuring the lesion size produced on the leaflets .

**DNA isolation**

For the genomic DNA isolation, the isolates of *C. cassiicola* were multiplied on PDA medium. Three mycelia plugs each of seven mm diameter were removed from five day old culture of each isolate and transferred to Potato Dextrose Broth (PDB). After five days of growth 3.0g of mycelium was harvested by filtering through sieve and transferred to sterilized mortar and was powdered in liquid nitrogen. The extraction and purification of total genomic DNA of *C. cassiicola* isolates were carried out by modified CTAB method (Doyle and Doyle, 1990; Roger and Bendich, 1994). The purity (quantity and quality) of the DNA obtained was checked using a nano drop spectrophotometer which gave the ngµl$^{-1}$ of DNA sample of each isolates.

**PCR Amplification**

Detection of the cassiicolin gene Cas 1 was conducted by PCR (Polymerase Chain Reaction) on genomic DNA from the isolates. The primers was designed from the reference sequence *Cas1* (EF667973) (Deon *et al.*, 2012a). PCR was performed in a 20µl reaction mix which contained 2 µl DNA sample, 2 µl 10X Dream Taq Buffer containing 20mM $MgCl_2$ (Thermospecific), 1.25 µl of dNTP from a 10mM dNTP mix, 0.17 µl Dream Taq DNA Polymerase enzyme (5Uµl$^{-1}$, 500 U), 2 µl each of Cas1 forward and reverse primers (Deon *et al.*, 2012a) and 12.58 µl Milli Q water.

The PCR was carried out in a Bio-Rad thermocycler. The PCR amplification profile consists of an initial denaturation step at 95 °C for 4 min followed by 35 cycles at 95 °C for 30 s, 52 °C for 30 s and 72 °C for 45s. After the cycles, a final extension step was given for 5min at 72 °C. On completion of PCR, 7 µl gel loading dye was added to each PCR tubes and the amplified products were analyzed along with a DNA marker (100bp) as molecular size references by electrophoresis on a 1.5 per cent agarose gel stained with Ethidium bromide in 1X TBE buffer. The gel was screened and visualized on a Gel Doc system (Biorad, USA). To confirm the reliability of the method, amplification reactions were performed 3-4 times.

## RESULT AND DISCUSSION

Colony morphology of *C. cassiicola* isolates cultured on PDA varied greatly. Differences were observed in the mycelial colour and texture as observed from the top of the plate and in colony colour when observed from the bottom of the plate (Table 1 and Fig. 1). The colony morphology of

Table 1. **Colony color, texture, and colony diameter of *C. cassiicola* isolates on potato dextrose agar measured after eight days incubation at room temperature.**

| Isolate | Colony colour | Colony texture | Colony diameter (cm) |
|---|---|---|---|
| Bathiyadukka | Medium grey | Cottony | 8.5 |
| Sheradi III | Cream | Cottony | 7.0 |
| Sheradi II | Cream | Cottony | 6.8 |
| *Corynespora* 19 | Dark grey | Cottony | 8.2 |
| Mullani | Medium grey | Cottony | 7.0 |
| Nelliyadi | Cream | Cottony | 6.7 |
| Sheradi I | White | Cottony | 7.6 |

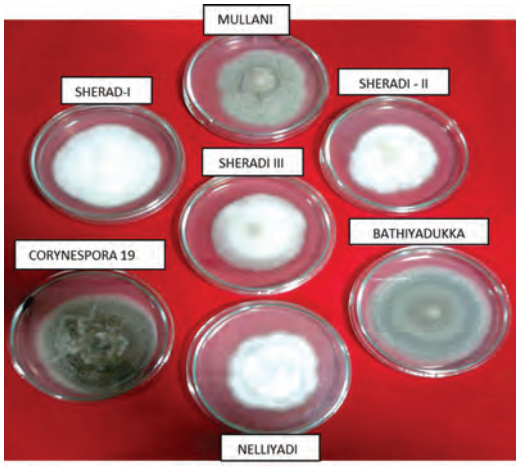



Fig.1. Colony morphology of *Corynespora cassiicola* isolates on PDA

*C. cassiicola* in this study showed high variation in colony diameter and colony colour which did not associate with geographic origin of the pathogens as described by Darmono *et al*. (1996).

In the leaf wilt bio-assay, isolates Sheradi-I, Sheradi- III and *Corynespora* 19 showed greater degree of aggressiveness when measured by the intensity of wilting of leaves in the susceptible clone RRII 105. Isolates Sheradi-II, Mullani and Nelliyadi showed moderate virulence and the isolate Bathiyadukka showed least virulence when compared with other isolates. On the other hand when considering the tolerant clone Fx 516, crude toxic fraction of *Corynespora* 19 showed notable wilting of the leaf when compared with the toxin produced by other *Corynespora* isolates, this is as Fx 516 is tolerant to the cassiicolin toxin when compared with the susceptible clone RRII 105 (Fig. 2 and 3 & Table 2). The cassiicolin produced by *C. cassiicola* isolates causes wilting of young susceptible leaves when inoculated in toxic fraction produced by different isolates.

Use of pure toxin to screen *C. cassiicola* has certain advantages over the direct use of fungal pathogen as the reaction of fungal infection is more sensitive to environment than reactions to toxic bioassays and the results of toxin bioassay can be obtained


Fig. 2. Leaf wilt bio-assay of clone RRII 105

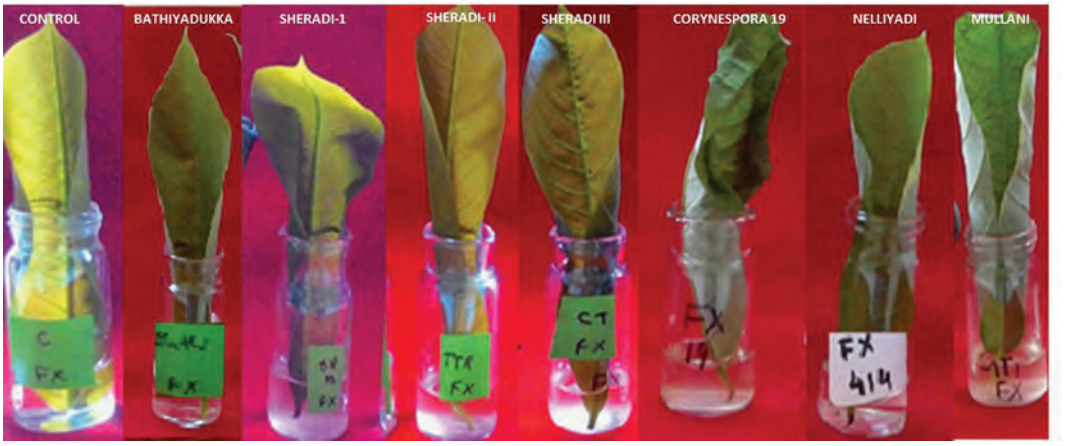



Fig. 3. Leaf wilt bio-assay of clone Fx 516

Table 2. ***In vitro* leaf wilt bioassay of clones**

| Isolate | RRII 105 | Fx 516 |
|---|---|---|
| Bathiyadukka | + | _ |
| Sheradi III | +++ | _ |
| Sheradi II | ++ | _ |
| *Corynespora* 19 | +++ | + |
| Mullani | ++ | _ |
| Nelliyadi | ++ | _ |
| Sheradi I | +++ | _ |

within 24 hour and hence more number of clones can be tested in a short time. Countries that are not affected by *C. cassiicola* can screen their clones to *Corynespora* disease without introducing fungus. Difference in the isolates can be studied through exchange of toxin preparation and raising antibodies against cassiicolin by eliminating the risk of introducing new strain of the fungus (Breton *et al.*, 2000).

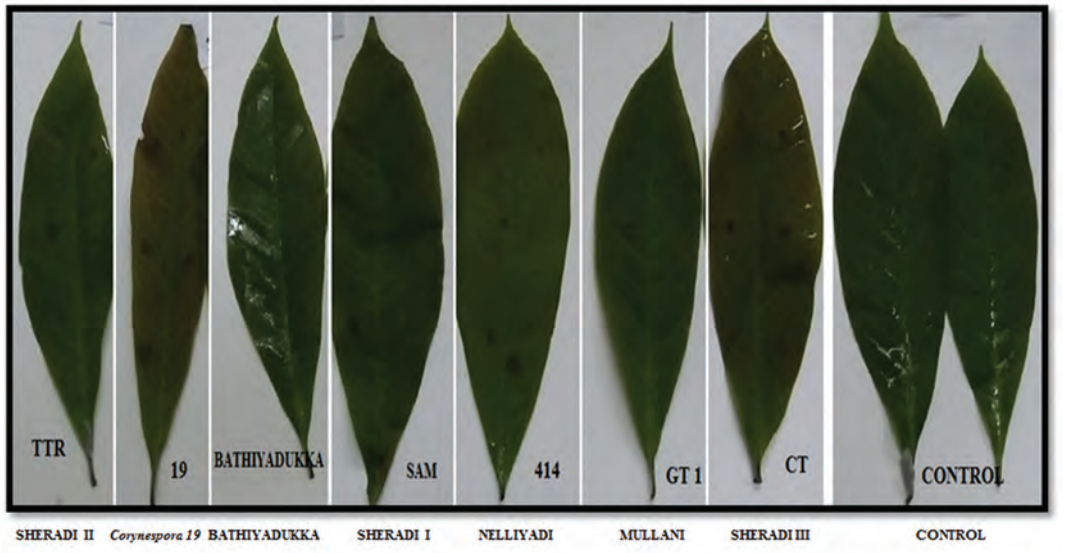



Fig.4. Effect of spore inoculation on clone RRII 105

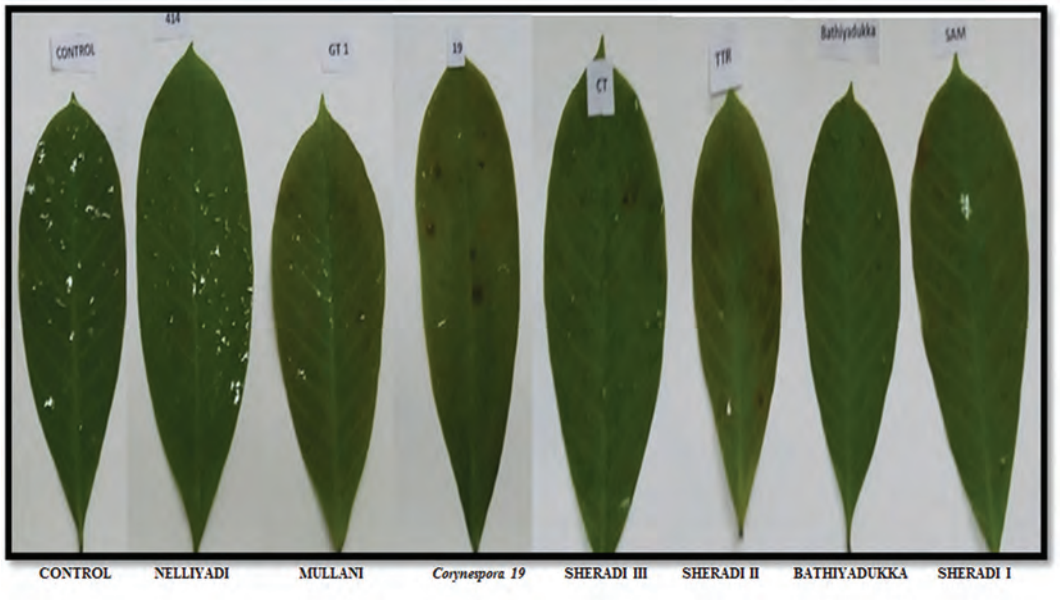



Fig.5. Effect of spore inoculation on clone Fx 516

Table 3.**The lesion size produced by spore inoculation of various *Corynespora cassiicola* isolates**

| Isolate | Size of lesion (Cm) in Clone RRII 105 | Size of lesion (Cm) in Clone Fx 516 |
|---|---|---|
| Bathiyadukka | 0.1 | 0 |
| Sheradi III | 0.6 | 0.1 |
| Sheradi II | 0.6 | 0 |
| *Corynespora* 19 | 0.8 | 0.7 |
| Mullani | 0.2 | 0.1 |
| Nelliyadi | 0.4 | 0 |
| Sheradi I | 0.2 | 0 |

In the spore inoculation bio-assay, toxic activity was estimated by measuring the lesion size produced on the leaflets. Sheradi-II, Sheradi-III and *Corynespora* 19 showed higher degree of virulence which was observed from the lesion size produced by the spore inoculation of these isolates on the susceptible clone RRII 105 (Fig. 4.) Bathiyadukka showed negligible lesion on the leaf of RRII 105 while it did not produce any lesion on Fx 516. Notable lesion on the excised leaf of clone Fx 516 was produced by *Corynespora* 19 (Fig. 5 and Table 3). Both the *in-vitro* bioassays play an important role in the identification of the races of fungus.

When comparing the leaf wilt bioassay with the spore inoculation method the isolates Sheradi-II, Sheradi-III and *Corynespora* 19 showed higher degree of aggressiveness in the susceptible clone RRII 105, while other isolates were moderately aggressive with an exception of the isolate Bathiyadukka which was least pathogenic when compared with the other six isolates under study. In the tolerant clone Fx 516, the isolate *Corynespora* 19 showed notable wilting and lesion in leaf indicating its high aggressiveness compared to other isolates, the isolate Bathiyadukka was the least aggressive isolate with low wilting and lesion size on rubber leaves.

The genomic DNA of *C. cassiicola* was quantified by measuring optical density

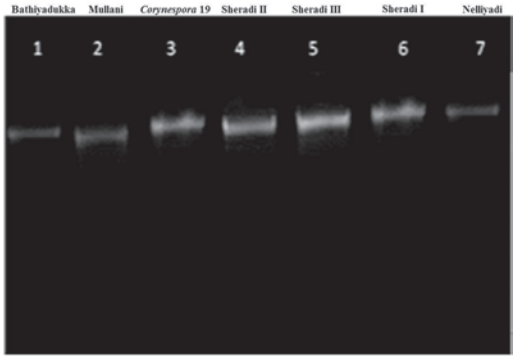
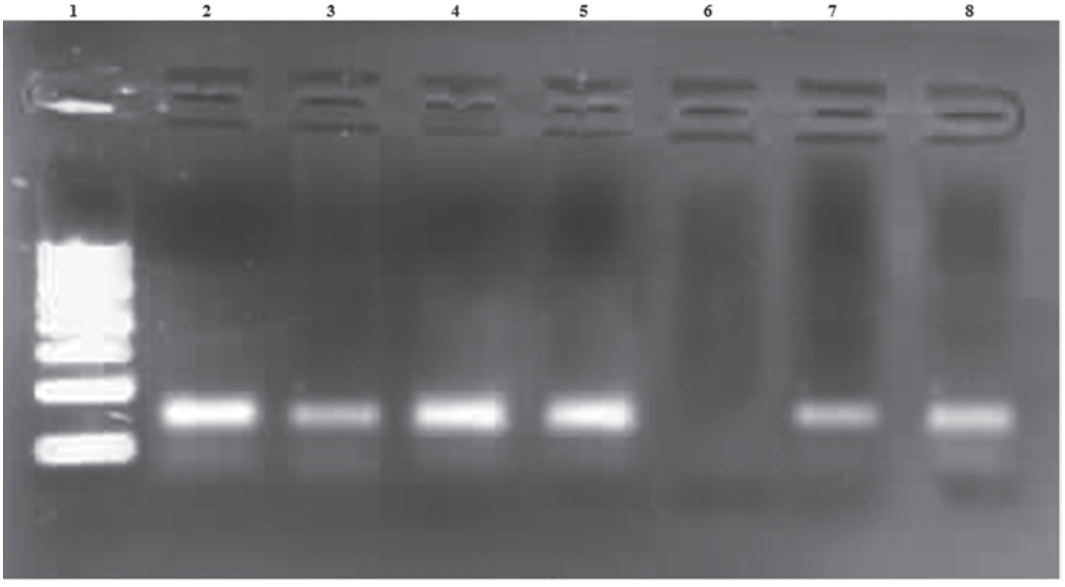



Fig. 6. Genomic DNA of seven different isolates of *Corynespora cassiicola*

(OD) at $A_{260}/A_{280}$ with a Nanodrop Spectrophotometer (ND 1000). The reading at 260 nm allows calculation of the concentration of the nucleic acid in the sample. An OD of 1 corresponds to approximately 50 µgml$^{-1}$ for single-stranded oligonucleotides. A ratio between 1.7:1.8 indicates good quality DNA without protein contamination. The concentrations of DNA samples of seven different *C. cassiicola* were quantified in a Nanodrop-1000 spectrophotometer. The isolated DNA was subjected to agarose gel electrophoresis and the bands were visualized in the Gel doc system (Biorad, USA) (Fig. 6)

The isolated genomic DNA was used to carry out PCR. Concentration of DNA is a critical factor to get better PCR amplification. PCR of the cassiicolin encoding gene *Cas1* was carried out using a set of primers (Deon *et al.* 2012a). Among the seven isolates, six of them revealed the presence of *Cas1* gene. The primers failed to amplify any band in Bathiyadukka which showed the absence of *Cas1* gene (Fig. 7.)

The isolate which do not amplify Cas gene has been classified as Cas0. The




Lane 1: 100bp Molecular Marker, Lane 2: Sheradi I, Lane 3: Mullani, Lane 4: Sheradi III, Lane 5: Sheradi II

Lane 6: Bathiyadukka, Lane 7: Nelliyadi, Lane 8: *Corynespora* 19

Fig. 7. PCR amplification of *Cas1* gene of seven different isolates of *Corynespora cassiicola*

different toxin classes were defined by the cassiicolin deduced protein isoforms (Deon *et al*. 2014). However, it may also indicate that the Cas genes have been acquired very recently (potentially through horizontal transfer) by some isolates, which therefore share close genetic backgrounds with isolates deprived of *Cas* gene. When relating Cas genes diversity to pathogenicity profiles in rubber tree, Cas1 isolates were found the most aggressive. It was previously demonstrated that the Cas1 gene is functional and transiently expressed in the early phase of the infection and that differences in aggressiveness within the isolates could be related to differences in the *Cas*1 gene expression (Deon *et al.*, 2012a).

From the PCR amplification carried out to detect the presence of cassiicolin coding Cas1 gene, the presence of Cas1 in the isolates Sheradi-I, Sheradi-II, Sheradi-III, Mullani, Nelliyadi and *Corynespora* 19 suggests its importance in early phase of infection and pathogenesis. The absence of Cas1 in Bathiyadukka suggests the least virulence of the isolate which was observed by leaf wilt bioassay and spore inoculation method. The cassiicolin genes contribute significantly to the overall genetic constitution of *C. cassiicola*, suggesting that these genes play an important role in the biology and evolution of the fungus. There may be yet uncharacterized effector molecules that may have a role in aggressiveness of the *Corynespora* isolates.

## CONCLUSION

*Corynespora cassiicola* isolates collected from different locations varied in their virulence /aggressiveness and was directly linked to their toxin production. Among the seven isolates tested for the detection of *Cas 1* gene by PCR with *Cas1* gene specific forward and reverse primers, the Bathiyadukka the least virulent isolates lacked the same gene. This suggests possibility for the existence of uncharacterized effectors and co-existence various effectors within the same group. Further studies reveling genetic structure of *C.cassiicola* will help in predicting potential races which can be physiologically validated by testing several isolates per genetic group on a large number of rubber cultivars

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