BIOLOGICAL CONTROL OF *Corynespora cassiicola* CAUSING CORYNESPORA LEAF FALL DISEASE (CLF) ON RUBBER TREE BY *BACILLUS THURINGIENSIS* (T3)

Pham Thi Ngoc Giau[1] and Nguyen Bao Quoc[2]

[1] Rubber Research Institute of Vietnam
[2] Research Institute of Biotechnology and Environment, Nong Lam University

Abstract

Bacillus thuringiensis *(T3) isolated from foliage and sapwood of* Hevea brasiliensis *is demonstrated to have biological control potential against CLF disease caused by* Corynespora cassiicola*. Pathogenicity of* C. cassiicola *is caused by the toxin, cassiicolin. This toxin is encoded by cassiicolin gene (cas). Screening of cas genes was conducted with twenty* C. cassiicola *isolates from Vietnam e by using PCR method. Two isolates were classified as cas2 and the remaining as cas0.* C. cassiicola *isolate containing cas2 gene (CoryQN01) was inoculated on detached rubber leaves with and without the treatment of* B. thuringiensis *(T3) bacterial isolate at various time intervals. The results indicated that cassiicolin gene expression was down-regulated under treatment with* B. thuringiensis *(T3).*

Keywords: antifungal ability, Corynespora cassiicola*, cas gene,* Bacillus thuringiensis

INTRODUCTION

Corynespora leaf fall (CLF) is caused by *Corynespora cassiicola* (Berk. & Curt) Wei (Ellis and Holliday, 1971). CLF disease affects both mature and immature *Hevea brasiliensis* leaves.*C. cassiicola* was first isolated from rubber trees in Sierra Lone in 1986 (Liyanage et al., 1986), followed by India (Ramakrishnan and Pillai, 1961+), Malaysia (Newsam, 1961), Nigeria (Awoderu, 1969), Indonesia (Soepena, 1983) and Thailand (Chanruang, 2000). Corynespora leaf fall disease has become a threat to the natural rubber plantation industry by limiting rubber latex production in Asia and Africa.

In Vietnam, CLF was first detected in Lai Khe Rubber Experimental Station of Rubber Research Institute of Vietnam in August 1999 (Jacob, 2006). More than 3000 susceptible trees were also removed from different estates in Vietnam to avoid the danger of disease spread from affected trees (Dung, 2000).

A toxin named cassiicolin was purified and characterized from culture filtrate of a rubber tree isolate of C. cassiicola (CCP) (Breton et al., 2000). The cassiicolin-encoding gene was found to be transiently

expressed one or two days post-inoculation, suggesting a role in the early phase of infection. Six cassiicolin isoforms (cas1, cas2, cas3, cas4, cas5 and cas6) were identified by PCR-detection on a collection of *C. cassiicola* strains from various host plants and geographical origins (Déon et al., 2014).

In a previous study, *Bacillus thuringiensis* (T3) isolated from foliage and sapwood of *H.brasiliensis* was found to have antifungal activity (Minh et al., 2014). The aims of the present study were (1) to detect the cas gene in 20 *Corynespora cassiicola* isolates (2) to investigate the inhibitory activity of *B. thuringiensis* in *Hevea brasiliensis* against *Corynespora cassiicola;* and (3) to evaluate the expression of cas gene under the induction of the antifungal bacteria *B. thuringiensis*.

MATERIALS AND METHODS

Fungal Strains and DNA Extraction

Twenty isolates *C. cassiicola* were obtained from Plant Protection Division, Rubber Research Institute of Vietnam which originated from rubber plantations in Vietnam (Table 1). These fungal strains were grown on Potato sucrose agar at 28°C for 7 days Mycelia were harvested by scraping the fungal colonies with a glass side and homogenized in a mortar in the presence of liquid nitrogen. Genomic DNA was extracted from fungi by Gene JET Plant Genomic DNA Purification Mini Kit, (ThermoFisher Sciencific) according to the manufacture’s instructions. The DNA was stored at -20°C until use. Quantification was performed with a spectrophotometer (NanoDrop-Thermo) and 1% agarose gel.

Table 1. Origins and sources of *Corynespora cassiicola* isolates used in this study

No.	Isolate	Location	Host clone	Collection year
1	*Cory*LK01	Plot ST04 Lai Khe, Binh Dương	LH97/0703	2009
2	*Cory*LK03	Trial 2000 - Breeding division, RRIV, Binh Dương	LH99/383	2005
3	*Cory*LK04	Trial 2000 - Breeding division, RRIV, Binh Dương	LH99/757	2005
4	*Cory*LK05	Trial 2000 - Breeding division, RRIV, Binh Dương	LH99/648	2005
5	*Cory*LK09	Trial 2000 - Breeding division, RRIV, Binh Dương	LH99/090	2005
6	*Cory*LK10	Trial 2000 - Breeding division, RRIV, Binh Dương	LH99/112	2005
7	*Cory*LK16	Trial 2000 - Breeding division, RRIV, Binh Dương	LH99/638	2005
8	*Cory*LK17	Trial 2000 - Breeding division, RRIV, Binh Dương	LH99/347	2005
9	*Cory*LK18	Trial 2000 - Breeding division, RRIV, Binh Dương	LH99/84	2005
10	*Cory*LK19	Trial 2000 - Breeding division, RRIV, Binh Dương	LH99/679	2005
11	*Cory*LK20	Nursery garden, Breeding division, RRIV, Binh Dương	RRIV 3	2010
12	*Cory*LK21	Breeding project, Binh Dương	RRIV 1	2010
13	*Cory*TN01	Ben Cui plantation, Tay Ninh	PB 255	2009
14	*Cory*BL01	Plot 2, Binh Long, Binh Phuoc	RRIV 3	2009
15	*Cory*DP01	Plot 10, Tan Hung platation, Đong Phu	RRIV 4	2010
16	*Cory*DN01	Nursery garden, Dong Nai	RRIV 3	2009
17	*Cory*DN02	An Loc, Dong Nai	VM 515	2010
18	*Cory*QN01	Duc Phu plantation,Quang Nam	PB 260	2009
19	*Cory*DC01	Duc Co, Gia Lai	RRIM 600	2009
20	*Cory*KT01	Plot 10, Suoi Cat plantation, Kontum,	RRIV 4	2010

PCR Amplification

Detection of cassiicolin gene was conducted by PCR on genomic DNA from the *C. cassiicola* isolates. The primer sequences details are given in Table 2.

Table 2. List of primers used for detection of cassiicolin gene

Primers names	Sequences (5' – 3')	Target gens	Expected size (bp)
F18	CCCAAGATACATGTTTTGAATGT	cas1	700
R27	CCACACAAAGCAAGATACAGAATG AGC	cas1	700
F17	GGATTTGCCTGAGATCCTA	cas2	759
R24	CAAACAATGCTAACCAAACAAAC	cas2	759
F20	GTCGGCTAACTTGGGAAAAACTCT	cas3, cas4	774
R28	GCAGGAAGCAAAACACAGAACAAG	cas3 , cas4	774
F19	CGGGGAGGTATCAGGTGTGAGATA	cas5	706
R26	CAGAACAAGCCAAAAGAGAACTAC	cas5	706
F16	GCTTGATTTGCCTGTGAGATACT	cas6	764
R25	AAAACGATGCTAAACAAAAGGA	cas6	764
F1CasU1-2-6	CTCTGCTTTTGTAGCAGCCG	cas1, cas2, cas6	457-461
R1CasU1-2-6	GTGTGGTGTATATGTAGCGC	cas1, cas2, cas6	457-461
F1CasU3-4-5	TCCCTATCCTCATCTCGGC	cas3, cas4, cas5	418-419
R1CasU3-4-5	GTCCAGAATACTTGGTAGC	cas3, cas4, cas5	418-419

PCR were performed in mixtures containing 1 µl template DNA, 200µM each dNTP and 0.4 mM each primers. The PCRs were carried out in a final volume of 25µl. The amplification reactions were carried out in a PCR system Model Mastercyrcle nexus, Eppendorf, with the following parameters: 95°C for 1 min; 30 cycles of deneturation for 30s at 95°C, annealing for 30s at 55°C, primer extension for 1 min at 72°C (10 min in the final cycle). Amplification experiments were repeated at least three times.

Gel Electrophoresis

PCR products were analyzed by electrophoresis through a 1% agarose gel at 100V for 30 minutes. The DNA bands were viewed and photographed under UV light using a UV transilluminator. A 1-kb DNA standard ladder was used as a marker.

DNA products amplified by PCRwere sequenced by FistBase (Singapore). The DNA sequences were edited with BioEdit software. To verify that the amplifed DNAs were derived from the target cas gene sequences, a search of the Gen Bank DNA database was conducted by using BLASTn program on NCBI. A phylogenetic tree of the homologous

cassiicolin gene was constructed using MEGA 7 software by the Neighbour-Joining method.

Pathogenicity Test of *C. cassiicola*

C. cassiicola isolates were grown on PDA at 25°C for 3 days, followed by PSA for 5 days in darkness. The conidia were collected and resuspended in 10 ml sterile water supplemented with 0.02% Tween20. The suspension was then filtered through two layers of gauze and adjusted to $2x10^3$ condia/ml. The rubber leaflets of 10 – 15 days after budburst were suface-sterilised with ethanol 50 – 60%, followed with 0.2% sodium benzoate for 30 seconds, washed with sterile water and were dried on strile paper towels. The detached leaflets were placed in closed plastic boxes on wet paper towels to maintain high humidity with the abxial surfaces facing upward (Hieu et al., 2014). Nine leaflets were used per rubber clone (three leaflets per box). The leaflets were inculated by pipetting 10µl drops of the spore suspension of *C. cassiicola* onto six spots per leaflet and were placed under fluorescent light in an air conditioned room (25°C).

The symptom intensity was scored 1,3,5,7 and 9 days after incubation using the assessment method (Ismail and Jeyanayagi, 1999 , and Nghia et al, 2008). The severity of infection was scored as follows: 0 - no visible lesion; 1 - small dark discolouration below droplet; 2 - prominent large lesion without extended blackish veins, 3, prominent large lesion with extended blackish veins; 4, prominent large lesion with mycelium. Lesion sizes were determined using the APS Assess 2.0 program (APSnet, St. Paul, MN, USA).

Antifungal Activity of Bacteria

The antifungal ability of *B. thuringiensis* T3 strain was tested by agar well diffusion method against the test fungus *C. cassiicola*, 2 ml spore suspension ($1x10^6$ CFU/mL) of test fungus was spread over the agar surface of the plate by using a sterilized swap stick. Three spaced wells of 6mm diameter were cut into the agar and by using a sterilized tip 50µl bacterial supernatant (1 – $2x10^8$ CFU/mL) was poured into each well. The plates were incubated at 28°C for 5-7 days. Appearance of clear zone around the well was taken as evidence for antifugal activity (Kumar et al., 2009). As a control, fungi were grown in absence of bacteria.

In Planta Antifungal Activity of Bacteria on Detached Leaves

A 10 µl droplet of of the spore suspension of. *C. cassiicola* was deposited on each detached rubber leaves using micropipette. After it dried, the same amount of *B.thuringiensis* T3 was applied in the same spot. After the inoculation, leaves were incubated in moist plastic boxes. The infection was observed 9 days after inoculation.

Total RNA extraction, cDNA synthesis and semi-quantitive cas gene expresssion by Reverse Transcription (RT) – PCR

In vitro expression:

Mycelium from *C. cassiicola* cultures in CM broth was collected after 7 days.

In planta expression:

Leaf discs (1.77 cm^2) were collected from the spore-inoculated detached leaves and spore-inoculated treatment with *B. thuringiensis* (T3) described above 1, 3, 5, 7 and 9 days after inoculation (Déon et al., 2012b).

Collected samples were frozen and ground to powder in liquid nitrogen. The total RNA was extracted using Gene JET Plant Genomic RNA Purification Mini Kit,(Thermo Scientific) according to the manufacture's instructions. RNA quantification was carried out using a Nanodrop spectrometer using 2µl aliquots.

Removal of genomic DNA from extracted RNA was done using RNase-free DNase I (BioLabs). Contaminating DNA was removed from total RNA by using 2 U of DNase I. The reaction mixture was incubated for 30 min at 37°C, and the DNase I was inactivated by adding 1 µl of 0.5 mM EDTA to the mixture and heating at 75°C for 10 minutes.

A two-step RT-PCR method was used to measure cas gene expression The first strand cDNA was synthesized from 1µg RNA using the RevertAid H Minus First Strand cDNA synthesis Kit (ThermoFisher Scientific) according to the manufacture's protocol. The cDNA stock was stored at -20°C.

The sequences of the specific primes used for RT-PCR reactions are defined in Table 3. PCR were performed in mixtures containing 1 µl cDNA, 200µM each dNTP, 0.4 mM each primers. The final volume of 25µl was mainained. PCR were carried out with an intitial denaturing step of 95°C for 5 min, followed by 1 min denaturing step of 95°C, annealing for 1 min at 49°C and extension for 1 min at 72°C. Housekeeping gene Actin was used as a control for variations in the input of RNA [5]. To determine gene expression in a semi-quantiative manner, PCR was run with 22 cycles and amounts of gene products relative to actin estimated. The reaction conditions used for semi-quantitatie RT-PCR are listed in Table 4. PCR products were resolved by electrophoresis in 1.5% agarose gel and photographed using BioDoc-It Imaging System (UVP Inc., Upland, CA). The band intensities were estimated by Gel analyzer software. Final results are expressed as the ratio of cas gene PCR product to actin PCR product for each sample.

Table 3. Forward (F) and reverse (R) primers used in real-time PCR for the reference genes and for the cas genes.

Primer	Sequence	Gene	Size of product (bp)	Reference
Actin R	GAGTCCTTCTGGC CCATACC	Actin gen (Act-1)	115	Déon et al., (2014
Actin F	CCACCATGGGTAC GAGAAC	Actin gen (Act-1)	115	Déon et al., (2014
F17	GGATTTGCCTGAG ATCCTA	cas2	759	Déon et al., (2014)
R24	CAAACAATGCTA ACCAAACAAAC	cas2	759	Déon et al., (2014

Table 4. Reation conditions used for semi-quantitative RT-PCR

Primer	Denaturation	Anneling	Extension	Cycles
Cas2		30s at 47°C		22
Actin		30s at 51°C		30

RESULTS AND DICUSSION

Detection of Cassiicolin Gene

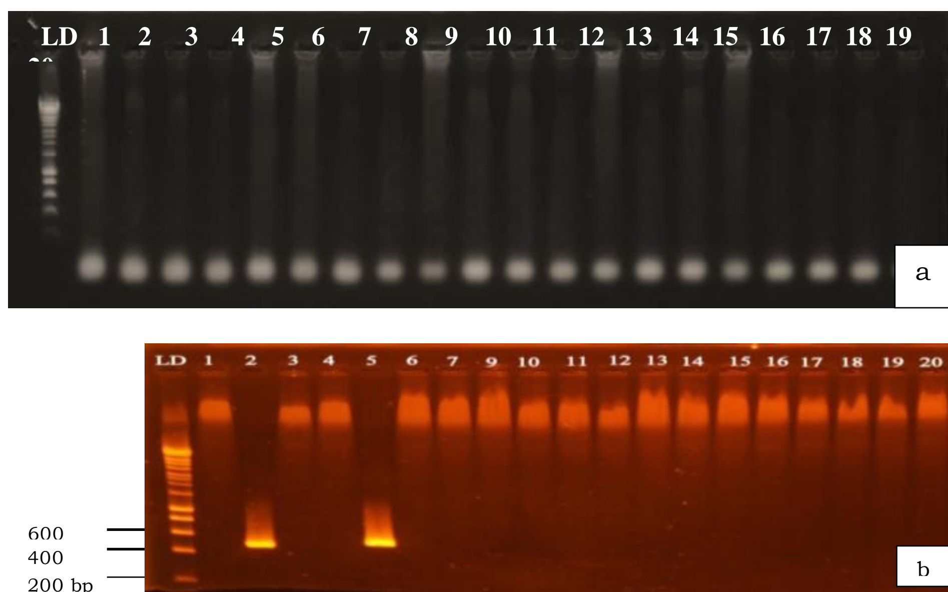


Figure 1. Agarose gel electrophoresis (1%) of PCR products obtained by (a) F1-R1CasU3-4-5 primer and (b) F1-R1CasU1-2-6. Lanes 1 to 20 LK03; QN01; DP01; KT01; DC01; LN02; BL01; DN01; DN02; LK17; LK18; LK19; LK20; LK21; TN01; LK01; LK04; LK05; LK09; LK16, respectively. LD: 1-kb DNA ladder

PCR was peformed on 20 isolates of *C. cassiicola* (Table 1) using the cas gen specific primers (Table 2). Fig 1a showed that no DNA amplication was observed in PCRs by universal primer F1-R1CasU3-4-5. The second PCR was performed using the universal F1-R1CasU1-2-6 primers. Fig 2a displays the amplification product with size around 450 – 500 bp in lane 2 and land 5, corresponding *Cory*QN01 and *Cory*DC01, respectively. No product was amplified from DNA of other isolates. This shows that the two strains *Cory*QN01 and *Cory*DC01 have the presence of cas1, cas2 or cas6.

To confirm that these two fungal isolates were cas1, cas2 or cas6, PCR with F18-R27, F16-R25 and F17-R24 specific primer for the cas 1, cas2 and cas6, respectively was performed. The PCR amplification products visualised by agarose gel electrophoresis are shown in Fig 2.

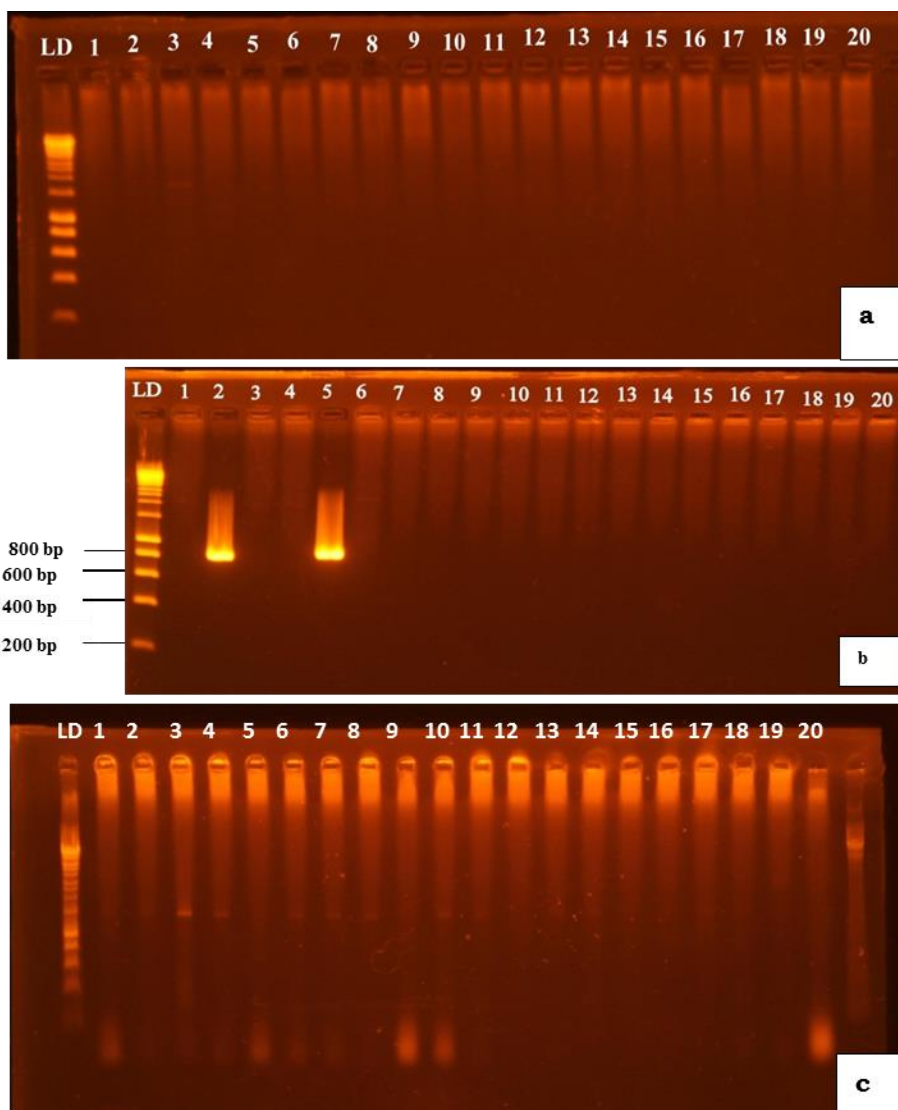


Figure 2. Agarose gel electrophoresis (1%) of PCR products obtained by (a) F18-R27 primer and (b) F17-R24 primer (c) F16-R25 primer. Lanes 1 to 20: LK03; QN01; DP01; KT01; DC01; LN02; BL01; DN01; DN02; LK17; LK18; LK19; LK20; LK21; TN01; LK01; LK04; LK05; LK09; LK16, respectively. LD: 1-kb DNA ladder

Fig 2a, 2c showed that no DNA products were observed in PCRs primed by cas 1 and cas 6. These results indicate that none of the isolates have cas1 and cas6 gen.

When PCRs were primed by F17-R24, two DNA fragments of approximately 750 bp, were amplified. (Fig 2b). DNA amplifications was

observed in lane 2 and lane5, corresponding *CoryQN01* and *Cory*DC01, respectively.

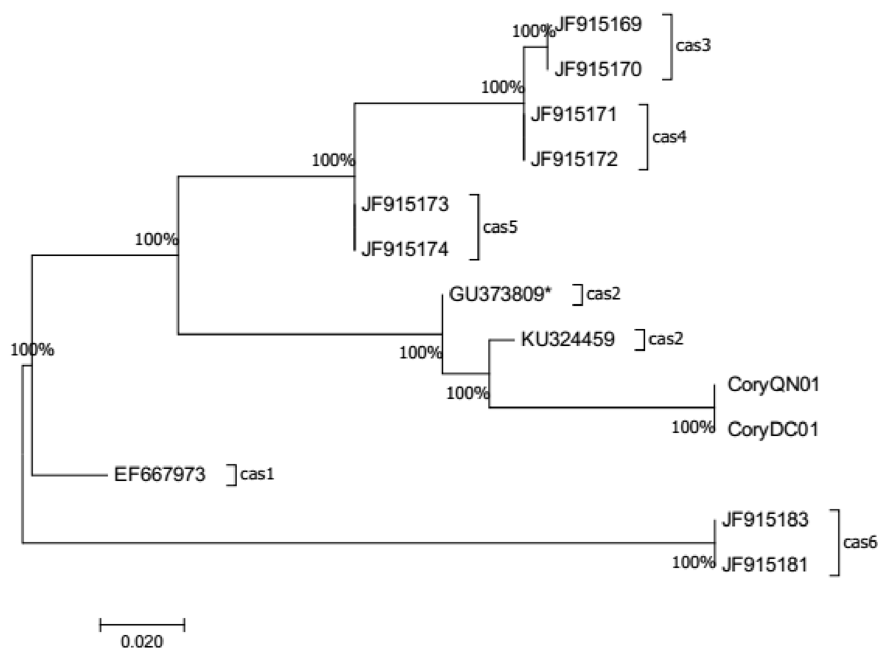


Figure 3. Molecular phylogenetic tree of the nucleotide sequences of the homologous cas gene and two sequences from *Cory*QN01 and *Cory*DC01 isolates. Sequences taken from GenBank are indicated by their accession numbers.

The phylogenic analysis involved 13 nucleotide sequences, two from our isolates and 11 of the homologous cassiicolin gene: cas1 (EF667973), cas 2 (GU373809; KU324459), cas3 (JF915169, JF915170), cas4 (JF915171, JF915172), cas5 (JF915173, JF915174), cas6 (JF915181, JF915183) from NCBI database. All positions containing gaps and missing data were eliminated. There were a total of 177 positions in the final dataset. The tree shows six groups corresponding to the six cas gene homologs. The isolates (*Cory*QN01, *Cory*DC01) belonged to the cas 2 homologues with high bootstrap values (100) (Fig 3).

Two of the twenty *C. cassiicola* isolates were classified as cas2. The other 18 isolates in which no cas gen was detected were classified as cas0 (Table 5). In a previous study by Liu et al (2015), the diversity of the cas gene was analysed in 49 *C. cassiicola* isolates sampled from various hosts and geographical origins in China. Most isolates(81%) had gene encoding a cassiicolin isoform, of which 6 isolates were with cas2 and 29

isolates with cas5 coding gene from rubber tree. Cas1, cas3, cas4 and cas6 were not found in any of the isolates.

Deon et al (2014) studied diversity of cassiicolin gene homologues of 70 *Corynespora cassiicola* isolates, 47% had at least one gene encoding a cassiicolin isoform. Some isolates in which no Cas gene was detected could, nevertheless, generate mild symptoms on rubber tree, suggesting the existence of other yet uncharacterized effectors.

Table 5. Results of detection cas gene by PCR

No.	Isolate name	Homologous cas gene					
		Cas1	Cas2	Cas3	Cas4	Cas5	Cas6
1	*Cory*LK01	-	-	-	-	-	-
2	*Cory*LK03	-	-	-	-	-	-
3	*Cory*LK04	-	-	-	-	-	-
4	*Cory*LK05	-	-	-	-	-	-
5	*Cory*LK09	-	-	-	-	-	-
6	*Cory*LK10	-	-	-	-	-	-
7	*Cory*LK16	-	-	-	-	-	-
8	*Cory*LK17	-	-	-	-	-	-
9	*Cory*LK18	-	-	-	-	-	-
10	*Cory*LK19	-	-	-	-	-	-
11	*Cory*LK20	-	-	-	-	-	-
12	*Cory*LK21	-	-	-	-	-	-
13	*Cory*TN01	-	-	-	-	-	-
14	*Cory*BL01	-	-	-	-	-	-
15	*Cory*DP01	-	-	-	-	-	-
16	*Cory*DN01	-	-	-	-	-	-
17	*Cory*DN02	-	-	-	-	-	-
18	*Cory*QN01	-	+	-	-	-	-
19	*Cory*DC01	-	+	-	-	-	-
20	*Cory*KT01	-	-	-	-	-	-

Pathogenicity on Detached Leaves on Two Rubber Clone, IAN 873 and RRIV 209

Two *C. cassiicola* isolates *Cory*DC01 and *Cory*QN01 were tested for pathogenicity on detached leaves of two *Hevea* clones IAN 873 and RRIV 209. The development of fungal infection was recorded 1, 3, 5 ,7 and 9 days after inoculation as described above.

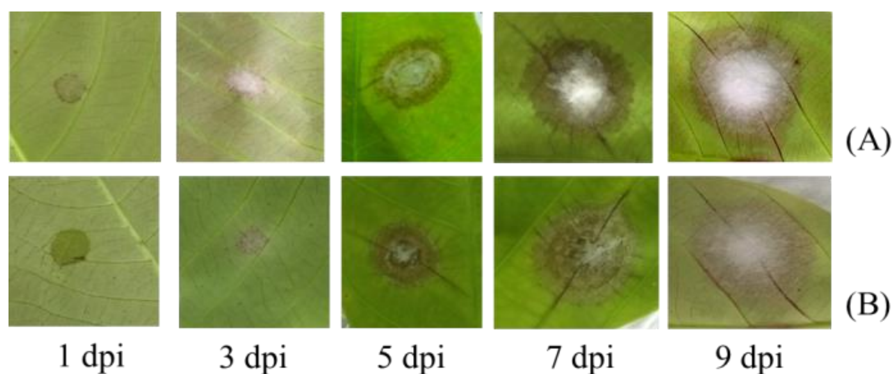
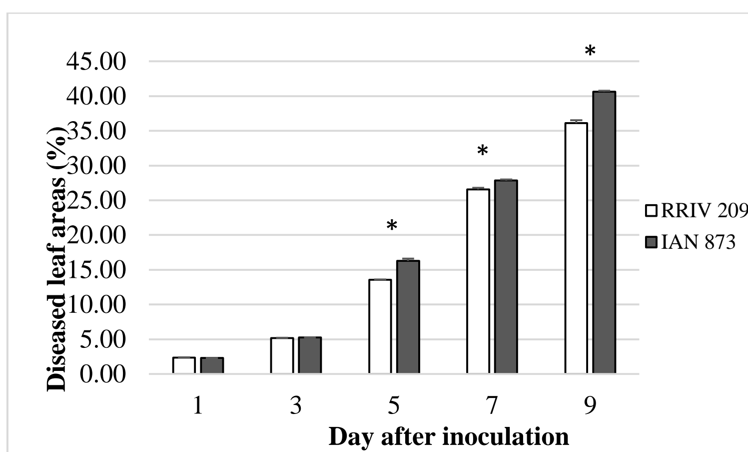


Figure 4. Pathogenicity of *Cory*QN01 isolate on detached leaves of (A) IAN 873 and (B) RRIV 209 *Hevea* clone



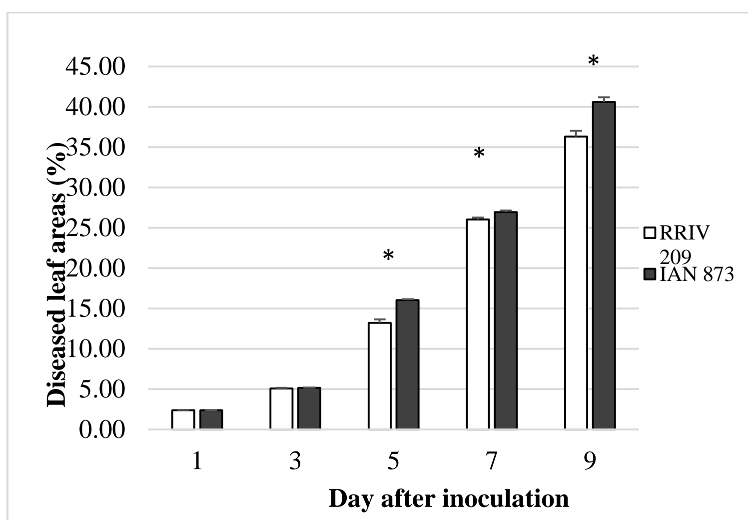


Figure 5. Assay for pathogenicity: percentage of infected areas of detached leaves (A) *Cory*QN01 (B) *Cory*DC01.(**, P<0,01, T-test)

As shown in Fig 4, 5, in the early days of infection (day 1 to 3), there was no significant difference between the diseased leaf areas (%) in the two rubber clones. Statistically significant difference was observed on day 5, day 7 and day 9 after infection. IAN 873 was more susceptible to *C. cassiicola* than RRIV 209. IAN 873 was selected for the antifungal resistance assay of bacterial isolates.

Antifungal Activity of *B. thuringiensis* (T3)

Antifungal Activity in-Vitro

The inhibition zone values for *Bacillus* T3 against *C. cassiicola* *Cory*QN01 and *Cory*DC01 are presented in Figure 6 and Table 6 . The results showed that the diameters of the inhibition zones for *Cory*QN01 and *Cory*DC01 isolates were 32.2 mm and 26.8 mm, respectively. Anova confirmed significant differences between the *Cory*QN01 and *Cory*DC01. *Cory*QN01 isolate was selected for the antifungal resistance assay of bacterial isolates.

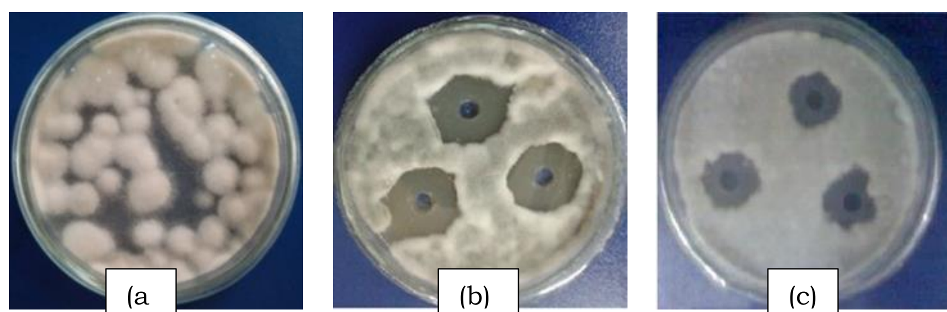


Figure 6. Antifungal activity of *Bacillus thuringiensis* T3 against *Cory*QN01 and *Cory*DC01 isolate by well agar diffusion method (a) control (b) *Cory*QN01 (c) *CoryD*C01

Table 6. Growth inhibition of C. cassiicola isolates by *Bacillus* T3 using well diffusion method.

Replicates	Diameter of the inhibition zones (mm)	
	*Cory*QN01	*Cory*DC01
1	31,6±1,5	27,2±0,3
2	33,3±0,8	26.2±0,3
3	31,8±1,1	26.9±0,2
Mean ± SD*	**32,2 ± 0,8**	**26.8 ± 0.4**

*Data are presented as the means ± SD from 3 independent experiments.

In Planta Antifungal Activity of Bacteria on Detached Leaves

Detached rubber leaves were inoculated with the pathogen *C. cassiicola*. The *B. thuringiensis (*T3) were then applied above the infected leaves. As shown in Fig 7, expanded necrotic lesions were observed in the infected untreated leaves (control)after nine days. In contrast, the lesion expansion were inhibited in infected leaves that were treated with *B. thuringiensis* (T3). The *B. thuringiensis* also inhibited the establishment of myceliumand the percentage of infected areas was reduced compared to that in the untreated leaves (Fig 8). l.

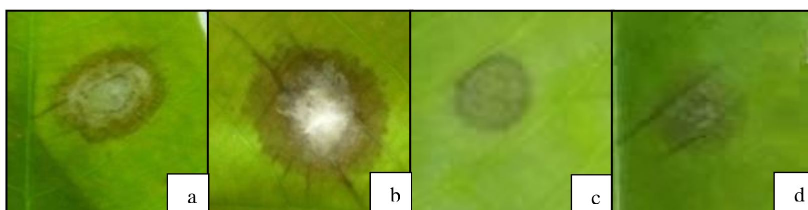


Figure 7. Antifungal activity of culture filtrate of bacteria on detached leaves (IAN 873) (a) control – 5dpi; (b) control – 9dpi; (c) treated with batecria – 5dpi; (d) treated with bacteria – 9dpi

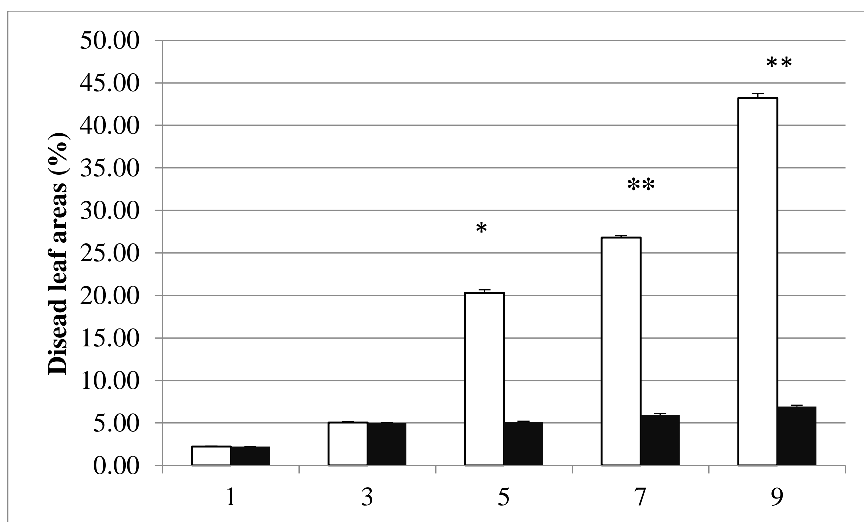


Figure 8. Assay for pathogenicity: percentage of infected areas of detached leaves treated with and without *B. thuringiensis* (T3). Error bars indicate standard deviation from the mean of 3 independent experiments (*, P < 0.01; T-test)

Bacillus sp. were found to inhibit the growth of *C.cassiicola* in *in-vitro* (Manju et al., 2014). Several studies have described the antifungal activity of endophytic bacteria. Most of the endophytic bacteria have antagonistic action against fungal pathogen. Endophytic bacteria inhibit growth of pathogens by production of antimicrobial compounds like antibiotics (Leyns et al., 1990).

Semi-Quantitive RT-PCR to Dectect Gene Expression Change

Semi-quantiative RT-PCR was used to detect gene expression changes in response to antifungal bacteria. For understanding if *B. thuringiensis* (T3) bacteria can inhibit the expression of cassiicolin gene (cas) involved in the release of toxin in plant cells, inoculation induced by *C. cassiicola* isolate containing cas 2 gene (*Cory*QN01) was done on detached rubber leaves with (treated samples) and without the treatment of bacterial isolate (control samples) at various time intervals including 1,3,5,7 and 9 days after inoculation. Cassiicolin gene expression was analyzed in spore-inoculated detached leaves and in mycelium produced *in vitro.*

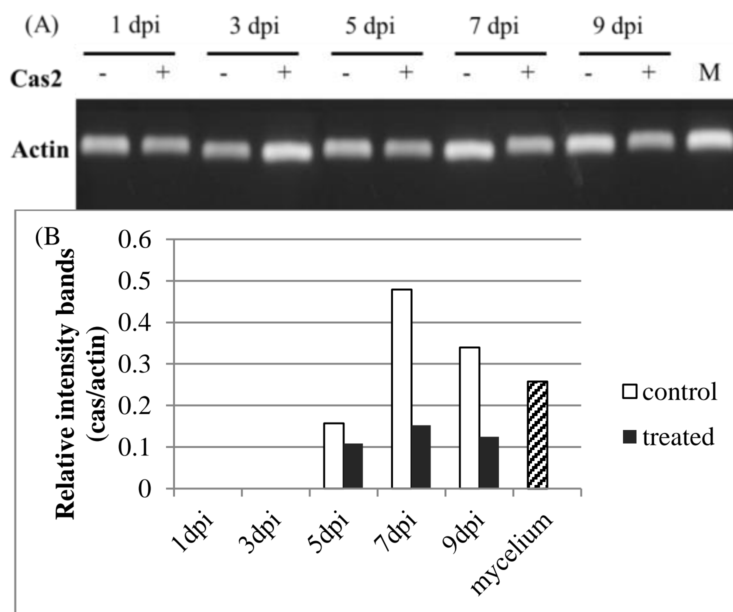


Figure 9. Semi-quantitative RT-PCR analysis comparing the cas gene expression in control and treated with T3 in 1,3,5,7 and 9dpi (day post inoculation). (A) representative lanes of RT-PCR products for cas and actin gene, infected detached leaves untreated (-) and treated (+) with *B. thuringiensis* T3 (B) RT-PCR products band intensity of the gel shown in Fig. 9A. Actin gene was used as an internal control.

As shown in Fig 9A, no amplicons were detected in control (untreated) and treated samples from infected detached leaves in the 1dpi and 3 dpi (Lane 1 – 4). The actin gene expression show no difference between the treated and control samples. The intensity obtained for bands in each lane (Fig. 9A) was normalized with the density obtained for the actin band and plotted (Fig 9B). In 5,7 and 9dpi, the treated samples bands was of lower intensity compared to control samples (Lane 5 – 11). The above results indicate that cas gene were significantly down-regulated in leaves treated with *B. thuringiensis* T3 compared to that of the control. In addition, the expression of the cas2 was observed only in the late stages of infection (5 – 9dpi).

The cassiicolin gene expression have been studied earlier (Deon et al., 2012a, 2012b). These studies showed that the gene encoding the cassiicolin precursor (Cas1, EF667973) is transiently expressed one or two days after inoculation, suggesting a role in the early phase of infection (Déon et al. 2012a). Unlike Cas1, Cas3 and 4 were not expressed after inoculation of the corresponding isolates on detached rubber tree leaves (Déon et al. 2012b). It has been demonstrated that the cas gene expression were different in cassiicolin gene homologues.

The cassiicolin gene was expressed *in vitro* (Lane 12) as well as *in planta.* These results consistent with the finding of Déon et al that cassicollin gene expression does not require plant factors to be turned on.

CONCLUSION

B. thuringiensis was found to inhibit the growth of the *C. cassiicola* in *in-vitro* and *in-planta*. Semi-quantitive RT-PCR was performed to compare the expression profile of cas2 gene on *C. cassiicola* infected rubber leaves under the treatment of B. thuringiensis(T3). The results indicated that cassiicolin gene expression was down-regulated under treatment with *B. thuringiensis* T3. Further studies conducted on whole plants are necessary for confirming the antifungal activity of *B. thuringiensis* T3 to *C. cassiicola*.

ACKNOWLEDGEMENT

We would like to acknowledge Dr Nguyen Anh Nghia (Rubber Research Institue in Vietnam) for providing the *C. cassiicola* isolates. We thank Mr Nguyen Van Minh (Open University Ho Chi Minh city) for providing the *B.thuringienis* T3 strain. We acknowledge Dr Valéria

(CIRAD, France) for kindly providing primer sequences used in this study for the detection of cassiicolin gene.

REFERENCES

Awoderu, V. (1969). New leaf spot of para rubber (Hevea brasiliensis) in Nigeria. *Plant Disease Reporter*, 53, 406-408.

Chanruang, N. (2000). Current status of Corynespora leaf fall in ThaiLand. Paper presented at the Presented at worshop on Corynespora leaf fall disease of *Hevea brasiliensis*, Kuala Lumpur, Malaysia and Medan, Indonesia, 6 - 15, June, 2000.

Déon, M., Bourré, Y., Gimenez, S., Berger, A., Bieysse, D., De Lamotte, F., & Oliver, G. (2012a). Characterization of a cassiicolin-encoding gene from *Corynespora cassiicola*, pathogen of rubber tree (*Hevea brasiliensis*). *Plant science*, 185, 227-237.

Déon, M., Fumanal, B., Gimenez, S., Bieysse, D., Oliveira, R. R., Shuib, S. S., & Seguin, M. (2014). Diversity of the cassiicolin gene in *Corynespora cassiicola* and relation with the pathogenicity in *Hevea brasiliensis*. *Fungal biology*, 118(1), 32-47.

Déon, M., Scomparin, A., Tixier, A., Mattos, C. R., Leroy, T., Seguin, M., & Pujade-Renaud, V. (2012). First characterization of endophytic *Corynespora cassiicola* isolates with variant cassiicolin genes recovered from rubber trees in Brazil. *Fungal diversity*, 54(1), 87-99.

Ellis, M. & Holliday, P. (1971). *Corynespora cassiicola* [target spot]. CMI descriptions of pathogenic fungi and bacteria.

Hieu, N. D., Nghia, N. A., Chi, V. T. Q., & Dung, P. (2014). Genetic diversity and pathogenicity of *Corynespora cassiicola* isolates from rubber trees and other hosts in Vietnam. *J Rubber Res*, 17(3), 187-203.

Ismail, H. & Jeyanayagi, I. (1999). Occurrence and identification of physiological races of *Corynespora cassiicola* of Hevea. Paper presented at the Proceedings of the IRRDB Symposium.

Kumar, A., Saini, P., & Shrivastava, J. (2009). Production of peptide antifungal antibiotic and biocontrol activity of *Bacillus subtilis*.

Leyns, F., Lambert, B., Joos, H., & Swings, J. (1990). Antifungal bacteria from different crops. *Biological control of soil-borne plant pathogens*, 437-444.

Liu, X., Li, B., Cai, J., Li, C., & Huang, G. (Eds.). (2015). Diversity of the cassiicolin gene in *Corynespora cassiicola* of the rubber tree in China, Proceeding International Rubber Conference 2015, International Rubber Conference 2015, Vietnam.

Manju, M., Benagi, V., Shankarappa, T., Kuruvilla Jacob, C., & Sabu, P. Antifungal activity of some biological agents against *Corynespora cassiicola* causing Corynespora leaf fall disease of rubber (*Hevea brasiliensis* Muell. Arg.).

Minh, N. V., Phuc, M. H., Yen Nhi, V. N., Linh, D. N., & Nghia, N. A. (2014). Screening of endophytes from rubber trees (*Hevea brasiliensis*) for biological control of *Corynespora cassiicola*. *TAP CHI SINH HOC*, 36(1se), 173-179.

Newsam, A. (1960). Plant pathology division report. Rubber Research Institute of Malaysia, Kuala Lumpur, Malaysia, 63.

Ramakrishnan, T. & Pillay, P. (1961). Leaf spot of rubber caused by *Corynespora cassiicola* (Berk. & Curt.) Wei. Rubber Board Bulletin, 5(1), 32-35.

Sujatno, S. T. (2000). Corynespora leaf fall disease on Hevea rubber in Indonesia. Paper presented at the IRRDB Workshop on Corynespora leaf fall of rubber.