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First characterization of endophytic *Corynespora cassiicola* isolates with variant cassiicolin genes recovered from rubber trees in Brazil

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Abstract *Corynespora cassiicola* is the causal agent of Corynespora Leaf Fall (CLF) disease. CLF is one of the most important fungal diseases of rubber trees in Asia and Africa but disease outbreaks have not been reported in South America. Cassiicolin, a small cysteine-rich glycoprotein secreted by the pathogenic *C. cassiicola* isolate CCP, was previously identified as a potential disease effector in rubber tree. Recently, the cassiicolin-encoding gene (*Cas1*) was characterized and shown to be expressed in the early phase of infection. In this study, we investigated whether previously undetected strains of *C. cassiicola* are present in South American rubber plantations by examining the fungal endophyte population found in asymptomatic rubber tree leaves. Four isolates were identified as *C. cassiicola*. Genes encoding new forms of the cassiicolin precursor protein (*Cas3* and *Cas4*) were identified from these isolates.

Three of four isolates were able to induce symptoms on the cultivar they were isolated from in a detached leaf assay, with different kinetics and intensities. One isolate had the same pathogenicity profile as the pathogenic isolate CCP; the other two isolates developed symptoms late during the course of infection, suggesting saprotrophic capabilities. However, no *Cas3* or *Cas4* transcripts could be detected upon inoculation with the endophytic isolates, whereas the reference gene *Cas1* was expressed upon inoculation with the CCP isolate. This work demonstrated that *C. cassiicola* is present in South America in an endophytic form and that it may evolve from an endophytic to a saprophytic or even potentially pathogenic life style.

Keywords *Corynespora cassiicola* · Rubber tree · Endophyte · Brazil · Cassiicolin

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Introduction

Corynespora cassiicola (Berk & M. A. Curtis) C.T. Wei is an anamorphic *Ascomycota* fungus belonging to the *Dothideomycetes* and forming a separate phylogenetic clade among the *Pleosporaceae* with *Corynespora smithii* (Schoch et al. 2009). It has been found on leaves, stems, fruits and roots of more than 300 plant species primarily in tropical and subtropical areas (<http://nt.ars-grin.gov/fungalatabases/>; Farr and Rossman 2011). Principally described as a pathogen, it causes severe damage to economically important plants, including rubber tree, tomato, cucumber, cotton and soybean (Chee 1990; Koenning et al. 2006; Oliveira et al. 2006, 2007; Schlub et al. 2009). However, *C. cassiicola* isolates were also obtained from dead organic material (Kingsland 1985; Lee et al. 2004; Cai et al. 2006) and

asymptomatic tissues (Collado et al. 1999; Suryanarayanan et al. 2002; Gond et al. 2007; Promptutha et al. 2007), and were therefore designated as saprophytes and endophytes, respectively.

In the rubber tree, *C. cassiicola* has thus far been exclusively known as a necrotrophic pathogen that causes the Corynespora Leaf Fall (CLF) disease, which ranks among the most important fungal diseases in Asian and African rubber plantations. Initially, *C. cassiicola* was described as a minor pathogen capable of attacking only budwood or seedling nursery plants (Newsam 1960; Chee 1988), but in 1975, the first epidemic outbreak on a plantation scale occurred in Indonesia. In the 1980s, several other countries in Southeast Asia were severely affected by disease outbreaks and thousands of hectares of rubber trees were uprooted in Malaysia, Indonesia, Thailand and Sri Lanka (Liyanage et al. 1986; Pongthep 1987; Chee 1988). By the end of the 1980s, African countries were also affected by CLF. The disease severity further increased until several important rubber tree cultivars considered to be tolerant or resistant to CLF during the first epidemic in the mid 1980s succumbed to the disease (Jayasinghe and Silva 1996; Shamsul and Shamsuri 1996; Sinulingga et al. 1996; Wahounou et al. 1996). Currently, all Asian and African rubber-producing countries, which account for 98 % of the natural rubber production in the world (94 and 4 % for each continent, respectively), are affected by the disease resulting in considerable economic losses.

CLF is characterized by necrotic lesions that develop on both young and mature leaves and lead to extensive defoliation. The fungus typically causes areas of necrosis with a fish bone appearance due to the darkening of the veins adjacent to the lesions (Chee 1988; Liyanage and Liyanage 1986; Pongthep 1987). However, the symptoms vary depending on the age, type and location of the rubber tree (Jayasinghe et al. 1998). This symptom variability impedes diagnosis of the disease in a plantation. Additionally, *C. cassiicola* isolates within the same agroclimatic zone vary widely in morphology, colony color, growth, spore production, pathogenicity and genetic diversity (Darmono et al. 1996; Jayasinghe and Silva 1996; Breton et al. 2000; Atan and Hamid 2003; Romruensukharom et al. 2005; Dixon et al. 2009; Qi et al. 2009).

Colonization of the rubber tree tissues by *C. cassiicola* involves the secretion of phytotoxic molecules (Onesirosan et al. 1975; Liyanage and Liyanage 1986; Purwantara 1987; Nugawela et al. 1989; Breton et al. 2000). A toxin called cassiicolin was purified and characterized from the culture filtrate of a rubber tree isolate (CCP) from the Philippines (Breton et al. 2000; Barthe et al. 2007; de Lamotte et al. 2007). The toxin is a small, secreted glycosylated protein that plays an important role in *C. cassiicola* pathogenicity. The cassiicolin-encoding gene encodes a precursor protein

containing a signal peptide at the amino terminus that is predicted to target the protein for secretion (Déon et al. 2012). This gene is expressed one or two days post-inoculation, suggesting a role in the early phase of infection.

Only a single report mentions CLF symptoms on *Hevea brasiliensis* growing in the American continent (Junqueira et al. 1985). In this area, *C. cassiicola* remains benign on rubber trees but causes significant damage to many other plant species. Could outbreaks of CLF disease occur in South American rubber plantations? To answer this question, we investigated whether previously undetected strains of the pathogen were present in rubber plantations in this area. The purpose of our study was to test for the presence of *C. cassiicola* among fungal rubber tree endophytes from a plantation in Brazil that had no history of the disease and to characterize these isolates.

Material and methods

Plant material

Fungal endophytes were recovered from young *Hevea brasiliensis* trees in nurseries consisting of 10 different cultivars (CDC 312, CDC 1174, FDR 5240, FDR 5665, FDR 5788, GT 1, MDF 180, PB 260, PMB 1 and RRIM 600) from a rubber tree plantation in Bahia, Brazil. The plants used for the inoculation and gene expression experiments (cultivars RRIM 600 and FDR 5788) were cultivated in a greenhouse in Clermont-Ferrand (France) at 28 °C±2 °C with 80 % relative humidity. All of the cultivars were grafted clones.

Isolation of endophytic fungi from asymptomatic Brazilian rubber tree leaves

Fungal endophytes were isolated from asymptomatic mature leaves that were collected in the nurseries and kept at room temperature for 8 days. Leaf segments were surface-sterilized through sequential immersion in 70 % ethanol (1 min), 2 % sodium hypochlorite solution (2 min), 70 % (v/v) ethanol (30 s) and sterile water. Leaf pieces with freshly cut edges were plated on Malt Extract Agar (MEA) supplemented with 0.02 % chloramphenicol and placed at 25 °C in the dark. The emergent fungi were isolated by successive subcultures.

Molecular identification of endophytic fungi

All fungal isolates were grown from single conidia and verified by sequencing the internal transcribed spacer (ITS) region of the ribosomal DNA. For DNA extraction the isolates were grown on Potato Dextrose Agar (PDA) for 13 days in the dark. The mycelia was collected, frozen in

liquid nitrogen and lyophilised. The genomic DNA was extracted as described previously (Risterucci et al. 2000). The ITS1, 5.8S, and ITS2 regions of the ribosomal DNA were amplified by PCR from 100 ng of genomic DNA in a 50 µl reaction mix containing 0.2 µM of the ITS1 and ITS4 primers (White et al. 1990), 200 µM of the dNTP mix, 2 mM of MgCl₂, 1× buffer and 1 U of Taq DNA polymerase (Qbiogen, Illkirch, France). The PCR was conducted for 30 cycles under the following conditions: 45 s at 94 °C, 45 s at 55 °C and 45 s at 72 °C. The PCR products were sequenced by GATC Biotech (Konstanz, Germany). Percentages of sequence similarity and coverage were determined by comparative similarity analyses with available sequences in the NCBI database using the Blastn program. Detailed results are given as Electronic Supplementary Material (ESM 1).

Detached-leaf assay

The *C. cassiicola* isolates were cultivated on PDA at 25 °C with a 12 h photoperiod. The conidia were collected and resuspended in sterile water supplemented with 0.02 % Tween20 at a concentration of 5000 conidia/ml. For each isolate, six leaves were inoculated, each with ten drops of 20 µl conidia suspension applied to the abaxial surface of detached rubber tree leaflets in developmental stage C (brownish to limp green) (Hallé and Martin 1968). One additional drop of 20 µl of sterile water supplemented with 0.02 % Tween20 was added to each leaflet as negative control. The leaflets were maintained in a moist environment at 25 °C for 24 h in the dark and then under alternate light with a 12 h photoperiod. The conidial suspension was evaporated four days after the inoculation. The lesion area per leaflet was measured manually, at 5 and 9 dpi. The entire experiment was conducted three times. The symptoms intensity (SI) was expressed as the mean lesion area ± the standard error from the 18 inoculated leaves (six leaflets per inoculation and three biological replicates).

Detection of cassiicolin gene homologues

Detection of cassiicolin gene homologues by PCR was conducted on the four *C. cassiicola* isolates (E70, E78, E79 and E139) from asymptomatic mature rubber tree leaves. The first set of primers was designed from the *Cas* sequence from isolate CCP (EF667973) and included CasF9, CasF11, CasF12, CasR16, CasR20 and CasR19. The second set of primers, CT1F9, CasF14, CT1R16 and CasR22, was designed from the CT1 sequence from the isolate CC004 (GU373809). Primer sequences are listed in the Electronic Supplementary Material ESM 2. PCR was performed on 100 ng of *C. cassiicola* genomic DNA for 30 cycles (45 s at 94 °C, 45 s at 50 °C, 45 s at 72 °C) using the same PCR components described above.

Cloning of full-length Cassiicolin gene homologues

The full-length sequence of the cassiicolin gene homologue *Cas3* was obtained by genome walking (Sallaud et al. 2003). This method allows for amplification of the 5' and 3' flanking regions of a target gene. Genomic DNA from isolate E70 was digested with 30 units of a restriction enzyme generating 3' blunt overhangs. Four restriction enzymes were tested independently: EcoRV, DraI, PvuII and StuI (New England Biolabs). The digested products were purified using the QIAquick PCR Purification Kit (Qiagen, Courtaboeuf, France) and ligated to the ADPR1/ADPR2 adaptor by T4 DNA ligase at 16 °C overnight in a final volume of 20 µl. The first PCR was performed with 1 µl of the ligation/digestion using the primer AP1, which is specific to the ADPR1 adaptor, and a primer specific to the *Cas3* partial sequence obtained previously from isolate E70 using the CasF9/CasR20 primer pair. The second PCR was performed with 2 µl of a 1/50 dilution of the PCR1 product using the nested primer AP2 (specific to the ADPR2 adaptor) and a *Cas3*-specific nested primer. To obtain the 5' flanking region, the primers AP1/CasR20 and AP2/CasW-E70-R04 were used for PCR1 and PCR2, respectively. To obtain the 3' flanking region, the primers AP1/CasF9 and AP2/CasW-E70-F04 were used for PCR1 and PCR2, respectively. PCR reactions were performed in 1× buffer containing 1.5 mM of MgCl₂, 200 µM of dNTPs, 200 nM of the adaptor, 0.2 µM of the *Cas*-specific primer and 0.5 U of *Taq* DNA polymerase (Eurobio, Courtaboeuf, France). All PCRs were conducted under the following conditions: an initial denaturation step (4 min at 95 °C), then 40 cycles (30 s at 95 °C, 30 s at 58 °C, 2 min at 72 °C) and a final extension step (72 °C for 5 min). PCR products migrating as a single unique band after electrophoresis on an agarose gel were directly sequenced using nested *Cas3*-specific primers: CasW-E70-R01 for the 5' flanking region and CasW-E70-F05 for the 3' flanking region. A new set of primers (CasF20 and CasR28) was designed from both ends of the 5' and 3' flanking sequences and used to amplify the complete *Cas3* or *Cas4* sequence from isolates E70, E78, E79 and E139 using the AccuPrime™ Pfx proofreading DNA polymerase (Invitrogen, Paisley, UK) according to the manufacturer's recommendations. All of the primers used in this study are listed in the Electronic Supplementary Material ESM 2.

Bioinformatics

All nucleotide and amino acid sequence analyses, alignments and annotations were conducted using the Geneious Pro program (Drummond et al. 2011). Homology searches were performed using the Blast program in the NCBI

database. A phylogenetic tree of the cassiicolin gene diversity was constructed using MEGA5 software (Tamura et al. 2007) by the Neighbor-Joining method (Saitou and Nei 1987). The analysis involved six nucleotide sequences: JF915169, JF915170, JF915171, JF915172, GU373809 and EF667973, for isolates E70, E78, E79, E139, CCP004 and CCP respectively. The codon positions included in the analysis were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 574 positions in the final dataset. A bootstrap test of 1000 replicates was performed to obtain the percentage in which the associated taxa clustered together (Felsenstein 1985). The evolutionary distances were computed using the p-distance method (Nei and Kumar 2000), and the results were expressed as the number of base differences per site.

The synonymous (d_S) and non-synonymous (d_N) substitution rates were calculated by codeml in the PAML package (Goldman and Yang 1994).

The prediction of the signal peptide in the protein was performed using SignalP software, version 3.0 (Bendtsen et al. 2004), and the program TMHMM, version 2.0, was used to check for the presence of transmembrane spanning regions in the protein (Krogh et al. 2001). The ProtComp program (version 9.0; <http://www.softberry.com>) was used to predict the subcellular localization of the protein.

Gene expression analyses by real-time PCR

RNA extraction and cDNA synthesis Leaf disks (1.77 cm²) were collected from the inoculated leaflets described above at each inoculation spot immediately after inoculation and then one, two, five and nine days post-inoculation. The controls were fragments from leaves inoculated with water supplemented with 0.02 % Tween20. For each time point, three sets of inoculated fragments were analyzed independently (three biological replicates). Collected samples were lyophilized and stored at -20 °C. The total RNA was extracted from the samples using CTAB extraction buffer (Chang et al. 1993), treated with RNase-free RQ1 DNase (Promega), quantified by spectrophotometry and quality tested by electrophoresis on 1.2 % agarose gels. The first-strand cDNA was synthesized from 1 µg of total RNA using oligodT and SuperScript III (Invitrogen) according to the supplier's protocol.

Design of Cas-specific primers Several pairs of primers were designed from the sequence of each *Cas* gene homologue, including at least one primer that overlapped an intron site. Their efficiency was tested on diluted cDNA pools of all time points for each isolate by cultivar set. The specificity of the amplification was analyzed using the melting temperature curves at the end of each run. The best

primer pairs were selected for the real-time RT-PCR experiments. The primers selected to amplify the *Cas1* transcripts were CasF12 and Cc-qCas1-R2. For *Cas3* and *Cas4* transcripts, the primers selected were Cc-qCas3,4-F1 and Cc-qCas3,4-R1. A third primer pair (Cc-qCas1,3,4-F1/Cc-qCas1,3,4-R1) designed to amplify conserved regions of all *Cas* homologue cDNA sequences was used as a positive control. All of these primer pairs failed to amplify any product from cDNA derived from non-inoculated leaves. Primer sequences are listed in the Electronic Supplementary Material (ESM 2).

Design of *C. cassiicola*-specific reference gene primers Primers were designed based on conserved regions (framing one intron site) determined from the alignment of *EF1α* or *actin* gene sequences from various fungal species, most of which belonged to the order Pleosporales, like *C. cassiicola*. Primers designed from the *EF1α* sequences were Nc-EF1α-F2 and Cc-EF1α-R1. Primers designed from the *actin* sequences were Cc-Actin-F4 and Cc-Actin-R1. These primers were used to amplify partial genomic sequences from all of the *C. cassiicola* isolates from this study. The PCR products were sequenced as described above and compared by multiple sequence alignment. New primers were designed for real-time RT-PCR, with the forward primer overlapping the intron. For *EF1α*, two forward primers were designed depending on the isolate due to a one-nucleotide substitution in the primer binding site. Primer Cc-qEF1α-F1 was developed for isolates CCP, E78, and E70 and primer Cc-qEF1α-F3 was developed for isolates E79 and E139. The reverse primer, Cc-qEF1α-R1, was the same for all isolates. For the *actin* gene, the primers designed were Cc-qActin-F2 and Cc-qActin-R2. The *actin* gene was used as a reference to validate the stability of the *EF1α* gene expression over the course of the infection (data not shown). The *EF1α* gene was used as a reference for the quantification of *Cas* gene expression. Primer sequences are listed in the Electronic Supplementary Material (ESM 2).

Quantification of the cassiicolin homolog transcripts by real-time RT-PCR Amplifications were performed using an iCycler IQ (Bio-Rad) with SYBR green as the fluorescent dye. The PCR reaction mix (25 µl) contained cDNA (2 µl of a 1/50 dilution of the first strand cDNA), 1× Mesa Green qPCR MasterMix Plus for SYBR Assay W/ fluorescein (Eurogentec, Angers, France) and 200 nM of each primer. Polymerase chain reactions were performed as follows: 3 min at 95 °C for denaturation and amplification for 40 cycles (10 s at 95 °C, 15 s at 62 °C, 15 s at 72 °C). The relative quantitative abundance (Qr) of the *Cas* homologue transcripts was calculated by comparison with the expression of *EF1α* using the following formula

(Pfaffl 2001), with E representing the primers' efficiency, "target" referring to the cassiicolin homologues and "ref" to *EF1 α* :

$$Q_r = \frac{(1 + E_{target})^{\Delta C_{t_{target}}}}{(1 + E_{ref})^{\Delta C_{t_{ref}}}}$$

The real-time PCR amplifications were performed in triplicate (technical replicates) and the experiment was repeated three times (biological replicates). Data presented are the mean \pm the standard error of the three independent biological replicates.

Monitoring of *C. cassiicola* development in lesions by real-time RT-PCR To analyze the development of the fungus in the plant tissues, the accumulation of transcripts of the *C. cassiicola*-specific *EF1 α* gene was monitored and compared to the expression of a *polyubiquitin* gene from the rubber tree (*Hb-polyubiquitin*, unpublished results). The primers used to amplify *Hb-polyubiquitin* transcripts were Hb-Ubi-F/Hb-Ubi-R (ESM 2). The composition of the real-time PCR mix and the program used for real-time PCR were the same as described above for the *Cas* homologues expression analysis, except for the annealing temperature (57 °C). The level of rubber tree colonization by *C. cassiicola* was represented by the relative expression (Q_r) of the fungal *EF1 α* gene normalized to the rubber tree *Polyubiquitin* transcript level.

Statistical analyses

Analyses of variance (ANOVA) were performed with software R, version 2.10.1 (R_Development_Core_Team 2009) and differences between means were tested using Tukey's Honest Significant Difference (HSD) test ($P < 0.05$). For real-time PCR, statistical analyses were performed on log-transformed data because empirical errors in Q_r increased with Q_r values consistent with the above exponential formulation.

Results

Diversity of the fungal endophytes

A total of 70 endophytic fungi were isolated from asymptomatic rubber tree leaves from a rubber plantation in Bahia, Brazil (ESM 1). The molecular identification based on ITS sequence similarity revealed that 100 % of the isolates were filamentous Ascomycota. Five genera predominated of which, 49 % of the isolates belonged to the genus *Colletotrichum* and its teleomorph *Glomerella*, 15 % to the genus *Phomopsis* genus and its teleomorph *Diaporthe*, 13 %

to the genus *Nigrospora*, 7 % to the genus *Xylaria* and 6 % to the genus *Corynespora*. Other rare genera were also isolated, such as *Guignardia* (two strains) and *Alternaria*, *Daldinia*, *Leptosphaerulina* and *Hypoxylon* (one strain each).

The four *Corynespora* isolates were identified as *cassii-cola* species, with at least 99.8 % identity and 100 % query coverage. *C. cassiicola* isolates E78, E79 and E139 were recovered from rubber tree cultivar RRIM 600 and isolate E70 was recovered from FDR 5788. This is the first report of an endophytic *C. cassiicola* in a rubber tree in Brazil. This is of significance as CLF disease outbreaks have not been reported in rubber tree plantations in South America, although *C. cassiicola* affects many other plant species in the area.

Description of new cassiicolin genes from *C. cassiicola* endophytic strains

The presence of *Cas* gene homologues in all four *C. cassiicola* endophytic strains was determined by PCR using different primer pairs designed from *Cas* (EF667973), the reference cassiicolin gene cloned from the rubber tree pathogenic isolate CCP originating from the Philippines (Déon et al. 2012), and CT1 (GU373809), a *Cas* gene homologue from a Chinese rubber tree isolate (CC004). Partial sequences were successfully amplified. The full-length sequence of the *Cas* gene homologues was obtained from all four isolates using the genome walking method. The new sequences were registered under the accession numbers JF915169, JF915170, JF915171 and JF915172 for isolates E70, E78, E79 and E139, respectively.

The nucleotide sequence alignment (ESM 3 and Fig. 1) revealed some diversity among the *Cas* gene homologues from the four endophytic strains, although they are closely related sequences. E79 and E139 *Cas* gene sequences were 100 % identical, while E70 and E78 *Cas* gene sequences shared 99 % identity with each other and 99 and 98 % identity, respectively, with the E79/E139 *Cas* gene sequence. Isolates E70, E78 and E79/E139 shared 78 %, 78 % and 79 % identity, respectively, with the reference

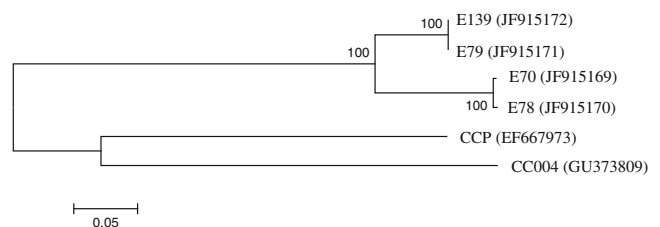


Fig. 1 Neighbor-joining phylogenetic tree of the cassiicolin precursor genes from four endophytic (E70, E78, E79 and E139) and two pathogenic strains of *C. cassiicola* (CCP and CC004). Bootstrap values are shown above the branch

Cas gene and 78 % identity with CT1. An alignment of the predicted amino acid sequences from all the *Cas* gene sequences revealed two new cassiicolin precursor proteins (Fig. 2). They were named Cas3 (protein id AFH88923 and AFH88924 from isolates E70 and E78 respectively) and Cas4 (protein id AFH88925 and AFH88926 from isolates E79 and E139 respectively), with Cas1 as the reference isoform (isolate CCP) and Cas2 as the protein encoded by CT1. Like Cas1, Cas3 and Cas4 are organized into an N-terminal domain including a 17 amino acid putative signal peptide followed by a 14 amino acid linker region and a 27 amino acid C-terminal domain corresponding to the putative mature protein. No transmembrane-spanning region was identified using the TMHMM program. An extracellular localization was predicted by Neural nets using the ProComp program, suggesting that the encoded protein may be secreted. Cas3 and Cas4 share 98 % identity (100 % positive amino acids) with each other, with only one substitution at position 15 in the signal peptide. They share respectively 93 % and 94 % identity (98 % positive amino acids) with the reference Cas1 sequence. The predicted mature cassiicolin domain shows one positive substitution (S instead of T) compared to the reference Cas1 sequence. Cas2 remains the most divergent protein isoform with seven substitutions and one insertion relative to Cas1, as described previously (Déon et al. 2012).

The 5' and 3' untranslated regions as well as the introns were the more divergent regions in the *cas* gene sequences. The ratio between the non-synonymous (d_N) and synonymous (d_S) substitution rates was calculated for each sequence pair to estimate the selection pressure acting on the *cas* gene. This ratio could not be calculated among the *C. cassiicola* endophytes since a single divergent nucleotide only was observed in their coding region. The d_N/d_S ratios calculated between the *cas* gene sequences from the isolates CCP, CC004 and the endophytes were all <1 (between 0.13 and 0.34) suggesting that the *Cas* gene may be under purifying selection pressure.

Pathogenicity of the *C. cassiicola* endophytes

Inoculations on detached leaves were performed to investigate the potential pathogenicity of the four *C. cassiicola* endophytic isolates on the cultivars from which they were

originally isolated (Fig. 3). The pathogenic strain CCP was used as a control on both cultivars. The water controls remained negative over the whole experiment. No necrosis was observed at 1 and 2 days post-inoculation (dpi) regardless of the isolate. At 5 dpi, only pinpoint necroses were visible on the leaves inoculated with the endophytic strains E78, E79 and E139 isolated from the RRIM600 cultivar. However, plants inoculated with the pathogenic isolate CCP had already developed disease symptoms at this time as lesion size had reached 445 mm². At 9 dpi, differences between isolates were evident and the pathogenic strain CCP was the most aggressive, followed by the endophytes E139 and E79. E78 remained under the virulence threshold (pinpoint necroses only). There was no significant difference in lesion size ($P < 0.05$) between the endophytic isolate E70 and the pathogenic isolate CCP on cultivar FDR 5788, with significant symptoms present at 5 dpi, which dramatically increased by 9 dpi.

Kinetics of mycelium development in the leaf tissues post-inoculation

The amount of mycelium that colonized rubber tree leaf tissue, post-inoculation was quantified by real-time PCR by calculating the relative expression (Qr) of a *C. cassiicola*-specific *EF1a* gene normalized to a rubber tree-specific *polyubiquitin* gene 1, 2, 5 and 9 dpi (Fig. 4). In the RRIM 600 cultivar (Fig. 4a), *EF1a* relative expression (Qr) was already detectable 1 and 2 dpi for E139 and CCP, while it was very low (nearly undetectable) for the other strains, suggesting that colonization of mycelia for these two strains started earlier, which is in agreement with their higher aggressiveness compared to E78 and E79. The Qr increased and reached a maximal level at 9 dpi, which was similar for both E139 and CCP. The development of E79 mycelium started later (between 2 dpi and 5 dpi) but finally reached levels similar to those of E139 and CCP at 5 and 9 dpi. In contrast, E78 mycelium colonization remained very low even at 9 dpi. In the FDR 5788 cultivar (Fig. 4b), the mycelium growth of both CCP and E70 was detectable as early as 1 dpi and strongly increased over time. Both strains presented similar profiles at 2, 5 and 9 dpi, although the mycelial growth may have started earlier for E70 than CCP.

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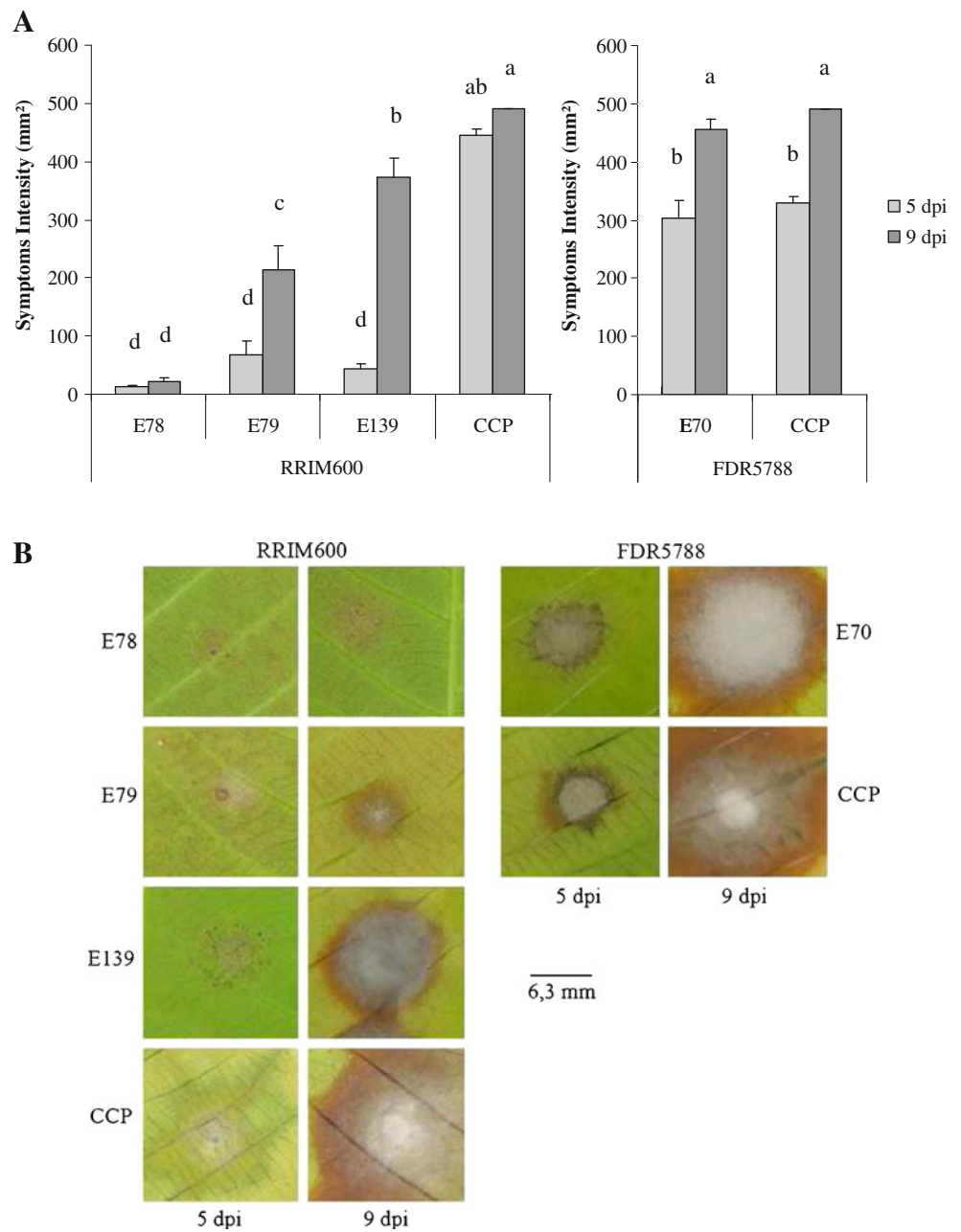
Cas1   MKYLPILISAFVAAVAAAPQDPS-AVAPVLPRTQCVSCVNFNGFGCGDNCGNSWACSGC
Cas2   MKYLPPIFISAFVAAVAAVPQGPSAAAAAILPRQSCVSCVDFGNGFGCGDNCGNSWACSGC
Cas3   MKYLPILISAFVAAITAAPQDPS-AVAPLLPRQSCVSCVNFNGFGFGDNCGNSWACSGC
Cas4   MKYLPILISAFVAAVTAAPQDPS-AVAPLLPRQSCVSCVNFNGFGFGDNCGNSWACSGC
*****:*****:*.**.**.*.*.:****:*****:*****

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Fig. 2 The amino acids sequence alignment of the cassiicolin precursor proteins Cas1 (ABV25895), Cas2 (ADC54229), Cas3 (AFH88923 and AFH88924) and Cas4 (AFH88925 and AFH88926). The mature cassiicolin domain is indicated by bold letters. The signal peptide is

underlined. CLUSTAL W annotation: conserved amino acids (*); amino acids of strongly similar properties (:); amino acids of weakly similar properties (.)

Fig. 3 Pathogenicity of four endophytic *C. cassiicola* isolates in a detached-leaf assay under controlled conditions. Isolates were inoculated onto the detached leaves of their respective original host rubber tree cultivar and pathogenic CCP strain was used as a control for both cultivars. For each isolate, six leaves were inoculated, each with ten drops of conidia suspension and one drop of water as untreated control. The lesion area per leaf was measured manually, at 5 and 9 dpi. The entire experiment was conducted three times. **Panel a:** Symptoms Intensity expressed as the mean lesion area \pm the standard error from the 18 inoculated leaves. For each cultivar, values followed by the same letter were not significantly different, according to Tukey's HSD test ($P < 0.05$). **Panel b:** Visual symptoms



Kinetics of *Cas* gene homologues expression post-inoculation

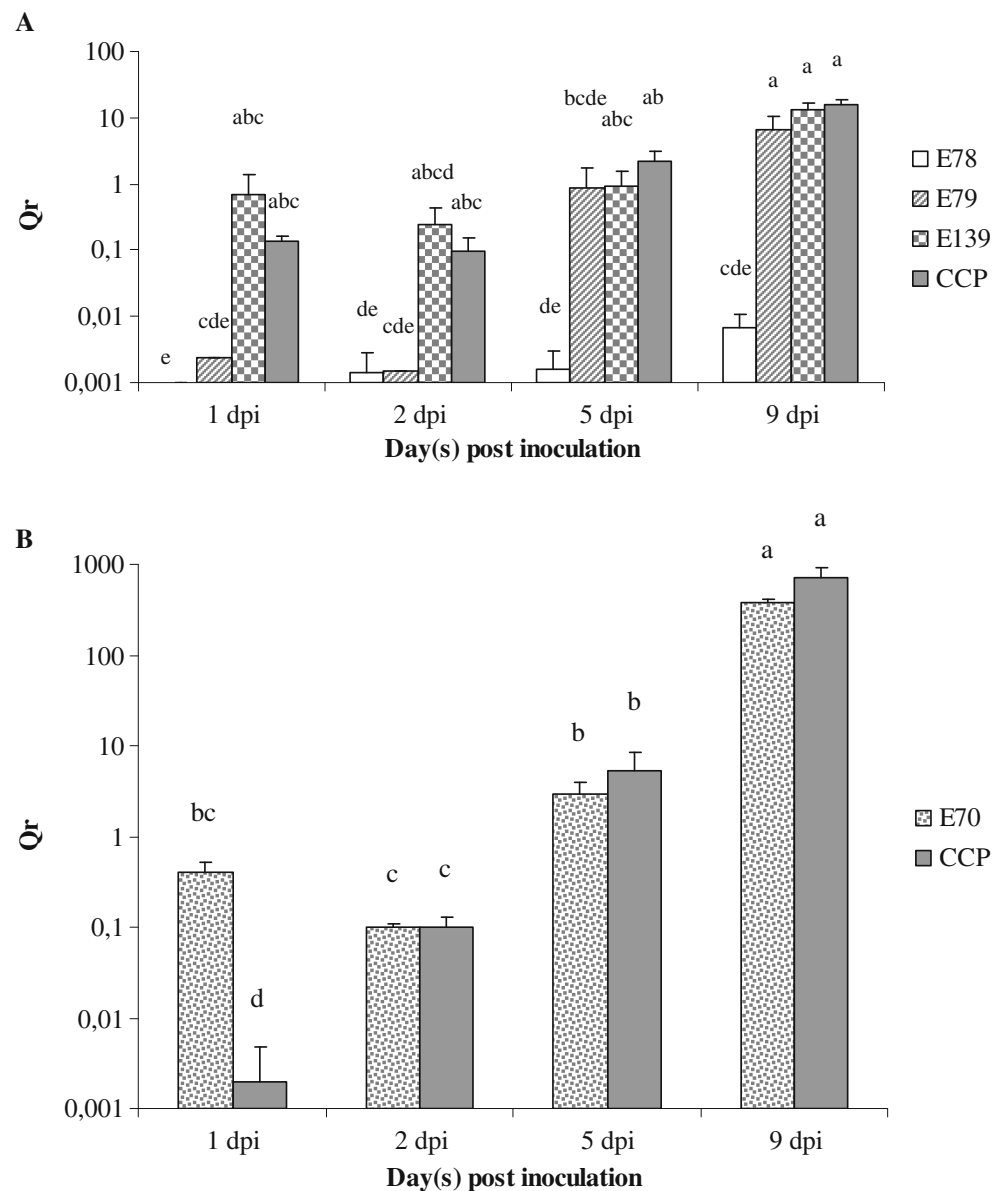
The relative expression level of the *Cas1*, *Cas3* and *Cas4* genes was determined at 1, 2, 5 and 9 dpi. Only *Cas1* from isolate CCP was expressed in RRIM 600 and FDR 5788 cultivars (Fig. 5). No *Cas3* or *4* transcripts were detected post-inoculation at any time point for any of the endophytic isolates. The *Cas1* expression profile in RRIM 600 was as expected based on previous analyses (Déon et al. 2012), with a transient peak of expression at 2 dpi. In FDR 5788, no peak of expression was observed and the *Cas1* relative expression remained similarly low at all time points.

Discussion

Diversity of the fungal endophytes in *Hevea brasiliensis*

There are still only a few studies investigating endophytic fungi in *Hevea brasiliensis*. The largest analysis was performed on wild rubber trees from Peru and compared the diversity of endophytic fungi in leaves and sapwood (Gaziz and Chaverri 2010). A second study was conducted on cultivated rubber trees from rubber plantations in Bahia, Brazil with the objective of identifying antagonists to *Microcyclus ulei*, another fungal pathogen of the rubber tree (Rocha et al. 2011). In our study, as in the study by Rocha et

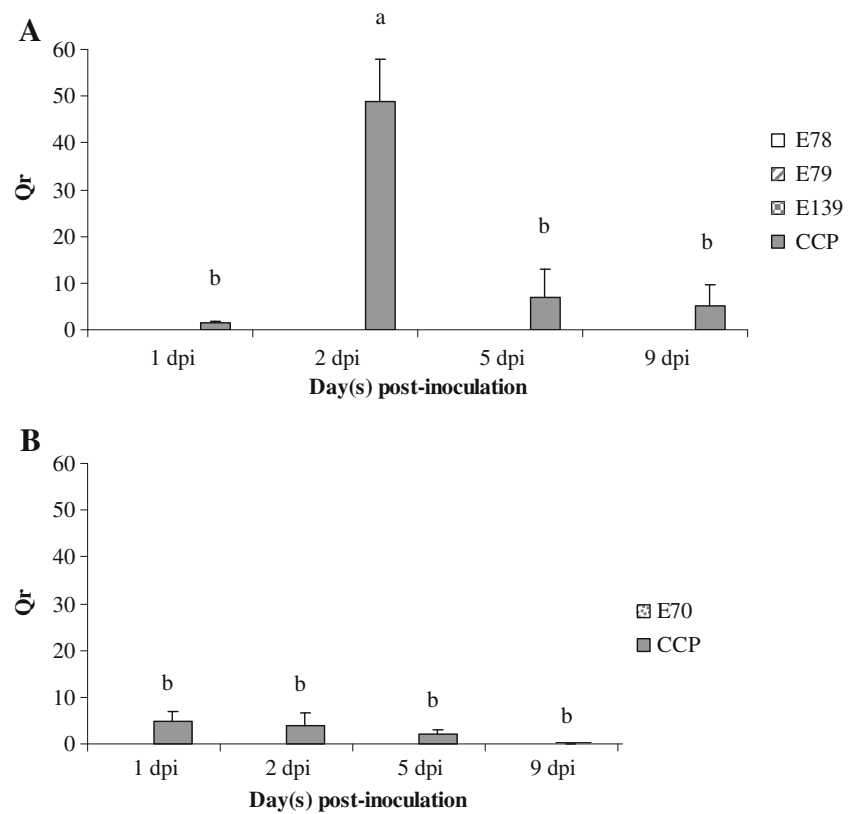
Fig. 4 Colonization of *C. cassiicola* mycelia in rubber tree leaf tissues post-inoculation measured by real-time PCR. The kinetics of *C. cassiicola* mycelia growth at 1, 2, 5 and 9 days post inoculation of the (a) RRIM 600 cultivar and (b) FDR 5788 cultivar were quantified by calculating the relative expression (Q_r) of a *C. cassiicola*-specific *EF1 α* gene normalized to a rubber tree-specific *polyubiquitin* gene. Data presented are means \pm standard error of three independent replicates. Values followed by the same letter were not significantly different according to Tukey's HSD test ($P < 0.05$)



al., all of the isolates identified were Ascomycetes. Gazis and Chaverri (2010) found that Ascomycetes were dominant (97 % of the isolates), but Zygomycota and Basidiomycota were also represented (2 % and 1 %, respectively), in agreement with the hypothesis that biodiversity is more important in the wild than in plantations. However, the identity and prevalence of the various isolated species varied among these three studies. In our study, the dominant genera were *Colletotrichum* (49 %), *Phomopsis* (15 %) and *Nigrospora* (13 %). Among these genera, only *Colletotrichum* and *Phomopsis* were found in all three studies. In the populations isolated from wild rubber trees from Peru (Gazis and Chaverri 2010), *Pestalotiopsis*, *Trichoderma* and *Penicillium* genera predominated (23 %, 22 % and 18 % of all isolates). Surprisingly, none of these genera were isolated in the course of this study or by Rocha et al. (2011). This could be explained

by the difference in geographical origin or cultivation history of the rubber trees. Gazis and Chaverri (2010) sampled wild rubber trees from the most biodiverse and undisturbed area of the world (Gazis and Chaverri 2010), while our study and Rocha et al. (2011) sampled rubber trees from plantations where biodiversity is clearly less important than in the forest. It should be underlined that Rocha et al. (2011) only provided molecular identification for the strains antagonistic to *Microcyclus ulei*, the causative agent of South American Leaf Blight (SALB), thus giving a partial representation of the endophyte population in the sampled trees. Nevertheless, three genera, *Fusarium/Gibberella*, *Myrothecium*, *Pestalotiopsis/Pestalosphaeria* and *Microsphaeropsis/Paraphaeosphaeria*, identified by Rocha et al. (2011) were not represented among our isolates even though the samples had the same origin of a rubber plantation in Bahia. The

Fig. 5 Real-time PCR analysis of *Cas* gene expression 1, 2, 5 and 9 days post-inoculation onto the (a) RRIM 600 cultivar and (b) FDR 5788 cultivar. Data presented are means \pm the standard error of three independent replicates. Values followed by the same letter were not significantly different according to Tukey's HSD test ($P < 0.05$)



physiological state of the leaves from which the endophytes were isolated, i.e. dry versus fresh leaves, could certainly have influenced the diversity of the recovered endophytic population. Among the specific genera that we found compared to Rocha et al. 2011, several species are known to degrade wood, such as *Xylaria sp.* or *Hypoxylon sp.* (Chaparro et al. 2009). This suggested that our study was selective for species associated with senescent plant material. Supporting this hypothesis, Promputtha et al. (2002) showed that the stage of leaf decomposition in *Magnolia liliifera* had an important impact on the diversity of endophyte populations.

An important result of our study is the identification of four *C. cassiicola* isolates. This is the first report of endophytic *C. cassiicola* in *Hevea brasiliensis*. *C. cassiicola* is primarily known as a pathogen affecting more than 300 plant species (<http://nt.ars-grin.gov/fungalatabases/> (Farr and Rossman 2011)). However, *C. cassiicola* was also reported as an endophyte of *Quercus ilex* (Collado et al. 1999), *Aegle marmelos* (Gond et al. 2007), *Magnolia liliifera* (Promputtha et al. 2007) and several other trees from tropical forests (Suryanarayanan et al. 2011). The fungus has also been observed as a saprotroph on cucumbers, tomatoes, papaya (Kingsland 1985), *Bambusa spp.* and *Dendrocalamus spp.* (Hyde et al. 2001), *Ischyrolepis subverticella* (Lee et al. 2004) and *Magnolia liliifera* (Promputtha et al. 2007, 2010; Kodsueb et al. 2008).

However, many other plants can support *C. cassiicola* growth as a pathogen, endophyte or saprotroph (Dixon et al. 2009). Our results demonstrate that, even though outbreaks of CLF disease have not yet occurred in South America, *C. cassiicola* is present in rubber trees on the American continent.

Are endophytic *C. cassiicola* isolates latent pathogens or latent saprotrophs?

Many species known to cause disease in plants are regularly isolated from asymptomatic tissues and are therefore also classified as endophytes (Kumar and Hyde 2004; Photita et al. 2004, 2005). Whether these are different subspecies or the same strain able to switch from one lifestyle to another is usually unknown. In the case of cacao (Rojas et al. 2010), haplotype subgroups were distinguished among *Colletotrichum gloeosporioides* isolates that were preferentially associated with either symptomatic or asymptomatic interactions. However, the isolates collected from asymptomatic tissues were not tested for pathogenicity. In several cases, endophytes inoculated onto their host plant under controlled conditions were found to be pathogenic (Mostert et al. 2000; Photita et al. 2004; Lana et al. 2011). With regard to *C. cassiicola*, Dixon et al. (2009)

showed that all isolates collected from healthy tissue of different plant species were pathogenic to the original host.

We inoculated four endophytic *C. cassiicola* onto detached leaves from their original host cultivar under controlled conditions. The strain E70 isolated from the FDR 5788 rubber tree cultivar induced symptoms when inoculated on the same cultivar, with virulence (Fig. 3) and mycelia colonization (Fig. 4) profiles similar to that of the pathogenic strain CCP. We may therefore wonder whether this endophytic *C. cassiicola* strain is a latent pathogen. This would be very worrying considering that rubber trees were so far spared from the CLF disease in this area. However, these experiments were conducted on detached leaves kept alive under moist environment for up to nine days, which cannot reflect exactly the field conditions. The initiation of the senescence process may have induced a lifestyle transition from endophyte to pathogen, in agreement with previous works showing that some endophytes may become pathogenic when the host plant is stressed (Fisher and Petrini 1992). However, a more probable interpretation would be that the observed symptoms reflect a saprotrophic process rather than parasitism.

Several studies proposed that fungal endophytes become saprotrophs when the host plants senesce (Promputtha et al. 2007, 2010; Okane et al. 2008; Porras-Alfaro and Bayman 2008). The close phylogenetic relationships between endophytes and saprotrophs isolated from healthy, mature and decaying leaves and twigs of *Magnolia liliifera*, including *C. cassiicola* isolates, suggest that these fungi have the ability to change their lifestyle during host senescence (Promputtha et al. 2007). This supports the concept of latent saprotrophism. Promputtha et al. (2010) demonstrated that a *C. cassiicola* endophyte and its saprobic counterpart, which was found during the middle to late stages (8–56 days) of leaf decomposition, were both able to produce laccase. The authors hypothesized that laccase activity from the *C. cassiicola* endophyte allows it to persist as a saprobe during decomposition.

In our study, the *C. cassiicola* strains isolated from asymptomatic rubber tree leaves were inoculated onto detached leaves from their original host cultivar, and the symptoms (necrotic surface area) and mycelium development were measured at various time-points from 1 to 9 days post-inoculation (dpi). This long kinetic revealed different phenotypes among the various isolates and suggested a possible switch from an endotrophic to a saprotrophic lifestyle. Indeed, the three endophytic isolates inoculated on RRIM 600 leaves were still under the virulence threshold at 5 dpi (the pathogenic strain had already induced significant symptoms at this time), but at 9 dpi, two of these isolates (E79 and E139) finally induced symptoms with significant mycelium development. It is possible that senescence-associated modifications of the leaf tissue enabled the penetration of

the mycelium inside the host cells and the saprotrophic development of these strains. It should be noted that some mycelium development could be detected by real-time RT-PCR prior to any visible necrotic symptom, as early as 1 dpi in case of E139, E70 and CCP. We suspect that these isolates may have a phase of epiphytic development before the mycelium penetrates through the cells upon toxin action (necrotrophy) or senescence-induced alteration of the tissues (saprotrophy). In the case of the isolate E78, which remained avirulent even at 9 dpi, we cannot rule out all saprobic activity but the very low amount of mycelium detected at 5 and 9 dpi demonstrated that it is clearly less competitive than the other isolates in senescing tissue.

Discovery of new cassiicolin gene homologues

New cassiicolin gene homologues potentially encoding two new cassiicolin precursor protein isoforms (Cas3 and Cas4) were found in the endophytic *C. cassiicola* isolates. Their predicted amino acid sequence is similar to that of the Cas1 reference isoform. In particular, the mature cassiicolin domain is highly conserved, with only one amino acid substitution (S instead of T) at position 2. This amino acid is especially important because it carries the sugar moiety (0-methyl-mannose) of the active cassiicolin (Barthe et al. 2007; de Lamotte et al. 2007). Although the role played by this sugar in toxicity is still unknown, it should be noted that Serine (S), like Threonine (T), can be 0-glycosylated. Therefore, the glycosylation of the toxin is not jeopardized by the T to S substitution.

The cassiicolin gene may be under purifying selection pressure, as indicated by the low (<1) d_N/d_S ratios. This suggests that this gene is playing an important functional role in *C. cassiicola*. However, this will have to be confirmed when a higher number of *Cas* gene sequences reflecting *C. cassiicola* evolution history will be available.

Although the genes encoding Cas3 and Cas4 appear structurally functional, no *Cas3* and *Cas4* transcripts could be detected post-inoculation. Therefore, if *Cas3* and *Cas4* genes are functional, it seems that their transcription is negatively controlled under the conditions used in this experiment. We have previously shown (Déon et al. 2012) that *Cas1* is transiently expressed, with a sharp peak of expression at 1 or 2 dpi depending on the cultivar. This was confirmed in this work for RRIM 600 inoculated with CCP. In the cultivar FDR 5788 inoculated with CCP, *Cas1* was expressed, but no peak of expression was observed. We suggest that the peak may have occurred at a different time-point not tested in this experiment. Whether *Cas3* and *4* can be switched on and under which conditions is unknown. In our analysis, some of the endophytic isolates induced necrosis on detached leaves five or nine days post-inoculation, although the corresponding *Cas* genes were not expressed,

suggesting that symptoms under these specific conditions are not mediated by the Cas3 or Cas4 proteins. This supports the idea that *C. cassiicola* can penetrate senescing tissues without the support of the Cas toxin and develop as a saprobe. The exact role of cassiicolin in the early phase of development and its ability to cause disease in intact plants needs to be further explored, over short time scales post inoculation.

Conclusion

In this work, we demonstrated that *C. cassiicola* is present in rubber plantations in Brazil in an endophytic form. Among the four isolates found, three were able to induce disease symptoms in a detached-leaf assay using rubber tree leaves under controlled conditions. This could be the manifestation of a saprotrophic lifestyle, although a pathogenic ability is not excluded, at least for one of the isolates. Whatsoever, our results suggest that the new *Cas* gene homologues identified in these isolates were not involved under the conditions used in this study. *C. cassiicola* affects many other plants in Brazil. It is possible that cassiicolin gene homologues play a role in other hosts and that their expression requires specific host plant signals. Rubber trees may serve as inoculum reservoir for these plants. Further studies conducted on whole plants are necessary to understand which parameters control *C. cassiicola* development and lifestyle. Potential antagonistic effects from other microorganisms should also be considered. The fungal endophytes isolated in this study in parallel with *C. cassiicola* are good candidates for antagonists to *C. cassiicola*. The exact role of cassiicolin and other potential effectors in the interaction between *C. cassiicola* and the rubber tree should also be investigated further.

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