Lívia Pimenta

Estudos bioquímicos e moleculares de *Ceratocystis* **spp. em** *Eucalyptus* **sp. e** *Arracacia xanthorhiza*

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Fitopatologia, área de concentração em Patologia Florestal, para a obtenção do título de Doutor.

Orientadora

Dra. Maria Alves Ferreira

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Eduardo Alves	UFLA
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Orientadora

LAVRAS-MG
2016

A Deus ,

AGRADEÇO

À memória do meu pai

À minha Mãe

Aos meus irmãos e ao Glauco

Pelo apoio e incentivo,

DEDICO

AGRADECIMENTOS

A Deus, pela fidelidade e por me dar força interior para superar as dificuldades.

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RESUMO

Espécies patogênica do gênero *Ceratocystis* têm ampla distribuição, principalmente nos trópicos, causando prejuízos em diversos hospedeiros de importância econômica. Em eucalipto, o uso de clones resistentes é a principal estratégia de controle de *Ceratocystis fimbriata*, porém pouco se conhece sobre as respostas de defesa envolvidas na resistência do eucalipto ao ataque do fungo. Nós observamos que *C. fimbriata* induziu respostas de defesa dos clones de eucalipto, independentemente do fenótipo resistente ou suscetível à doença. A quantidade de açúcares e lignina produzida pelo clone resistente (CR) inoculado com o fungo foi superior em comparação com o clone suscetível inoculado (CS), porém as atividades das enzimas polifenoloxidase e peroxidase foram maiores no CS. Quanto aos compostos fenólicos, a concentração de ácido hidroxinâmico do CR foi superior em comparação com o CS. Isoladamente, observou-se que o CR apresentou maior concentração dos compostos fenólicos catequina, ácido clorogênico e ácido cafeico. Nos testes histoquímicos, a presença de compostos fenólicos e lignina foram observadas em todos os tratamentos, sendo a maior intensidade detectada nas seções do CR. As alterações na concentração de açúcares e de lignina, bem como de algumas enzimas e compostos fenólicos podem ser úteis para predição do fenótipo de resistência de clones de eucalipto resistentes à doença. Em mandioquinha salsa (*Arracacia xanthorhiza*), onde fungo foi relatado recentemente, ainda não se conhece sobre variedades resistentes e a forma de controle do patógeno na cultura, ainda depende de outros estudos. Dessa forma, no presente trabalho foram realizados estudos filogenéticos, fertilidade, morfológicos e testes de patogenicidade do fungo na cultura e em inhame (*Colocasia esculenta*), onde o fungo causa, também, a podridão negra em tubérculos. As árvores da região ITS, *MAT1-1-2* e *MAT1-2-1* de isolados de *Ceratocystis* de mandioquinha-salsa agruparam em um clado separado dos outros isolados de *Ceratocystis*. A estirpe MAT2 masculina de

Eucalyptus não cruzou com a estirpe MAT1 do isolado de *Ceratocystis* de mandioquinha-salsa, o resultado do cruzamento foi a formação de alguns peritécios malformados, com hifas ostíolares deformadas, sem massas de ascósporos no topo do pescoço do peritécio. Os isolados de mandioquinha salsa foram, morfologicamente, semelhantes a outros isolados pertencentes ao complexo *C. fimbriata*, exceto pela presença de conídios cilíndricos com parede espessa. Os isolados também foram patogênicos a mandioquinha-salsa, porém não patogênico a inhame. A inoculação realizada em raízes de mandioquinha-salsa e tubérculos de inhame mostrou desenvolvimento e produção de estruturas fúngicas, no entanto, observou-se a formação de um halo avermelhado em tubérculos de inhame quando inoculadas com isolados de mandioquinha-salsa, mostrando que as respostas de defesa do hospedeiro podem estar presentes e bloqueando o ataque de patógenos. Com base em evidências filogenéticas, experimento de acasalamento, morfologia e especialização do hospedeiro, uma nova espécie associada a mandioquinha-salsa foi aqui descrita como *Ceratocystis arracacicola* sp.nov.

Palavras-chaves: Mandioquinha salsa, Eucalipto, murcha-de-ceratocystis, filogenia, compostos fenólicos.

ABSTRACT

Pathogenic species of the *Ceratocystis* genus are widely distributed, especially in the tropics, causing damage to several hosts of economic importance. In Eucalyptus, the use of resistant clones is the main control strategy for *Ceratocystis fimbriata*. However, little is known about the defense responses involved in Eucalyptus resistance to fungal attack. We observed that *C. fimbriata* induced defensive responses of the eucalyptus clones, regardless of the resistant or susceptible phenotype. The amount of sugars and lignin produced by the resistant clone inoculated with the fungus (RC) was higher when compared with the inoculated susceptible clone (SC), but the activities of polyphenol oxidase and peroxidase enzymes were higher in the SC. As for the phenolic compounds, the concentration of hydroxycinnamic acid in RC was higher compared with CS. Singularly, it was observed that the RC had higher concentration of phenolic compounds catechin, chlorogenic acid and caffeic acid. In histochemical tests, the presence of lignin and phenolic compounds were observed in all treatments, with the greatest intensity detected in the RC sections. The alterations in the concentration of sugars and lignin, as well as some enzymes and phenolic compounds might help predicting phenotypes of eucalyptus clones resistant to this disease. The number of pathogen hosts has increased in Brazil. Already in Peruvian carrot (*Arracacia xanthorhiza*), on which fungus has recently been reported, there are no known resistant varieties control method for the pathogen in this culture, which still depends on other studies. Thus, in this study, we performed phylogenetic and morphological studies, as well as, interfertility and fungal pathogenicity tests on this culture and on taro (*Colocasia esculenta*), to which the fungus causes also the black rot. The trees of the ITS region, MAT1-1-2 and MAT1-2-1 of *Ceratocystis* isolates of the Peruvian carrot, were grouped in a clade separate from other *Ceratocystis* isolates. The MAT2-male tester strains from eucalyptus did not successfully

cross with MAT1 tester strains of *Ceratocystis* isolates on Peruvian carrot. The result of the crossing was the occurrence of only a few malformed perithecia, with misshapen ostiolar hyphae without ascospore masses on the top of the perithecial neck. Pathogenicity tests indicated that the tested isolates were pathogenic to Peruvian carrot, but no symptoms were observed on taro plants, demonstrating host specificity. The inoculation performed on roots of Peruvian carrot and taro tubers, showed development and production of fungal structures. However, we observed the formation of a reddish halo on taro tubers when inoculated with isolates from Peruvian carrot, showing that host response may be present and blocking the pathogen attack. Based on the phylogenetic evidence, mating experiments, morphology and host specialization, a new species associated to Peruvian carrot was herein described as *Ceratocystis arracacicola* sp. nov.

Keywords: Peruvian carrot, eucalyptus, wilt Ceratocystis, phylogeny, phenolic compound

SUMÁRIO

PRIMEIRA PARTE

1. INTRODUÇÃO

Ceratocystis é um gênero que compreende espécies patogênicas para vários hospedeiros de importância florestal e agronômica. *Ceratocystis fimbriata* foi a primeira espécie descrita do gênero que é considerada, filogeneticamente, um complexo de espécies agrupadas em quatro clados distintos: o da América Latina, o da América do Norte, o da Ásia e o da África (HARRINGTON, 2000; HEATH et al., 2009; JOHNSON et al., 2005; THORPE et al., 2005). No clado da América latina, *C. fimbriata*, causa doenças em várias plantas lenhosas ou herbáceas como o eucalipto (*Eucalyptus* spp.), manga (*Mangifera indica*), seringueira (*Hevea brasiliensis*), figo (*Ficus carica*) e inhame (*Colocasia esculenta*) (BASTOS; EVANS, 1978; RIBEIRO; CORAL, 1968; MUCHOVEJ et al., 1978; VALARINI; TOKESHI, 1980; ALFENAS et al., 2009).

Em plantas lenhosas, geralmente, os sintomas se iniciam como murcha de galhos e ramos. Por ser um patógeno de xilema, o sintoma característico é observado nas secções transversais do lenho, na forma de estrias radiais escuras. Com o crescimento do fungo e a formação de aleurioconídios, os elementos de vasos são bloqueados, impedindo o transporte de água para parte aérea da planta, ocasionando o sintoma de murcha (BAKER; HARRINGTON, 2004).

Em eucalipto, o plantio de clones resistentes à *Ceratocystis* é a principal e única forma segura para o controle da doença (ZAUZA et al., 2004). Entretanto, a resposta da planta ao ataque do fungo é pouco conhecida. O que se sabe é que em eucalipto foi observado que a presença de *C. fimbriata* elevou o teor de extrativos solúveis orgânicos, inorgânicos e lignina (MAFIA et al., 2013). Em outras arbóreas, como plantas de *Platanus acerifolia* inoculadas com *C. fimbriata* f. sp. *platani*, também foi observado o aumento de compostos fenólicos (EL MODAFAR et al., 1993, 1996) e em cultivares de manga

(*Mangifera indica*) foram detectadas mudanças do sistema oxidativo da planta após inoculação com o fungo (BISPO et al., 2015). Em eucalipto, o conhecimento básico sobre os mecanismos bioquímicos envolvidos na resposta de defesa das plantas possui importância para o aprimoramento dos métodos de avaliação da resistência e para melhor embasamento dos programas de melhoramento genético.

Já, em tubérculos e raízes, o sintoma típico de *Ceratocystis* é a podridão negra, relatada, também, em inhame e batata doce (HARRINGTON et al., 2005; HALSTED, 1980). Na superfície de batata doce, por exemplo, sintomas de manchas escuras, formato circular e ligeiramente aprofundada podem ser observados. Dentro das manchas, são observadas estruturas do fungo (peritécio) com longos pescoços, visíveis a olho nu. A podridão negra, geralmente, permanece superficial, mas pode se estender para a parte interna da batata, levando ao apodrecimento por organismos secundários (HALSTED, 1980). A implementação de práticas culturais e sanitárias adequadas, são medidas preventivas que podem reduzir, significativamente, a ocorrência de podridão negra durante a colheita ou no armazenamento.

Em raízes de mandioquinha salsa, onde foi, recentemente, detectada a presença de *C. fimbriata* (MELO et al., 2015), ainda não foram estudadas as medidas de controle da doença. Além disso, considerando a evolução no conhecimento sobre a taxonomia desse e grupo de patógenos, torna-se importante confirmar a identificação da espécie com base em estudos filogenéticos, de fertilidade e outros. Esses estudos tornaram-se importantes, principalmente, após Ferreira et al (2010) propor que os isolados brasileiros formam uma única espécie de *C. fimbriata* devido à fertilidade entre os isolados brasileiros. Após Harrington et al. (2014, 2015) observarem problemas com a taxonomia de *C. fimbriata* quando as espécies foram delimitadas apenas utilizando sequências de ITS, e demonstrarem que isolados *Ceratocystis* de

inhame, romã e nêspera provenientes da China possuiam sequências da região ITS e do gene de acasalamento semelhantes as sequencias de isolados brasileiros de eucalipto. Oliveira et al. (2015) também verificaram que espécies anteriormente descritas com base em sequências de ITS (*C. manginecans*, *C. acaciivora*, *C. mangicola*, e *C. mangivora*) são consideradas sinônimos *C. fimbriata*.

O presente estudo foi dividido em dois artigos, o primeiro teve como objetivo avaliar as respostas bioquímicas em clones de eucalipto resistente e suscetível à *C. fimbriata*. No segundo artigo, objetivou-se estudar as características morfológicas, de patogenicidade, fertilidade e as relações filogenéticas de isolados de *Ceratocystis* obtidos de *A. xanthorrhiza* com isolados de outros hospedeiros bem como, verificar se o fungo se trata de uma nova espécie de *Ceratocystis*.

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SEGUNDA PARTE

Artigo 1: Biochemical responses of *Eucalyptus* spp. hybrids to infection by *Ceratocystis fimbriata*

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Abstract

In Brazil, the selection and plantation of eucalyptus clones resistant to *Ceratocystis fimbriata* is the preeminent control strategy of the disease. However, little is known about the defense responses associated with resistance of eucalyptus to *C. fimbriata* infection. In this study, enzymatic activity, sugars, lignin, total phenols, and phenolic compounds involved in the defense response of eucalyptus clones resistant (RC) and

susceptible (SC) to the Ceratocystis wilt were evaluated. Firstly, we detected changes in the sugar amount produced by RC clones, being superior compared to SC. Similar result was seen for lignin content at 28 days after inoculation (dai) in RC plants. Regarding the enzymatic activity, SC plants had an increase in the activity of polyphenol oxidase and peroxidase at 7 dai. In contrast to RC plants which demonstrated high activity of such enzymes in the firsts days, decreasing afterwards. There was no difference on phenylalanine ammonia-lyase activity between resistant and susceptible clones. The phenolic compounds, hydroxycinnamic acid concentration was higher in RC than in SC. However, there was no difference regarding flavonoids concentration. Singly, RC showed high concentration of the phenolic compounds: catechin, chlorogenic acid, and caffeic acid. Histochemical tests demonstrated presence of phenolic compounds and lignin in all treatments, with higher intensity, mainly, in sections of RC. *Ceratocystis fimbriata* inoculation induced defense responses in eucalyptus clones whether resistant or susceptible. Nevertheless, both intensity and speed of responses were higher in RC. The alterations in concentration of sugars and lignin, as well as some enzymes and phenolic compounds might help predicting resistant phenotypes of eucalyptus clones regarding this disease.

Key words: *Eucalyptus* spp., Ceratocystis wilt, disease resistance, defense mechanisms, phenolic compound

1 Introduction

Eucalyptus spp. plantations have high production of wood in Brazil. Due to a wide genetic variability and management technologies, it is possible to select plants and conduct plantings in different soils and climate conditions, enabling rapid biomass accumulation and provision of wood for several purposes. For this reason, nowadays, *Eucalyptus* spp. is the major cultivated forest species in the country. For instance, in 2014 the planted area totaled 5.56 million of hectares, with an increase of 1.6 % compared to the previous year. Such forestry base growth was pushed primarily by cellulose and paper segment (IBÁ 2015).

Eucalyptus expansion into new regions of Brazil has favored incidence of several diseases such as Ceratocystis wilt. This plant disease is caused by *Ceratocystis fimbriata* (Ellis & Halsted), which is a fungus of major importance in eucalyptus plantations because of its damages to the plants, besides of its difficulty to control. The fungus infects plants by penetration through roots or mechanic injuries caused during harvest or crop handling. As it attacks plant vascular systems, the initial symptoms emerge as wilting of branches and sprouts throughout the trunk (Ferreira et al. 2013). Internally in the trunk, fungal colonies develop dark radial

from the medulla, causing a vascular system darkening (Alfenas et al. 2009; Ferreira and Milani 2002).

Plants can provide several defense responses against fungus attack, as increasing contents of organic soluble extractives and lignin (Mafia et al. 2013). Studies on defense responses in trees species commonly report the production of phenolic compounds (Araújo et al. 2015; El Modafar et al. 1993, 1996).

Nowadays, selection and plantation of eucalyptus clones resistant to Ceratocystis wilt is the safest method to control the disease (Alfenas et al. 2009). However, little is known about biochemical mechanisms involved in the defense responses of eucalyptus clones to *C. fimbriata* infection. The knowledge about these mechanisms play important role to improve the methods of resistance evaluation methods and being the basis for eucalyptus breeding programs. In this perspective, this study assessed the contents of soluble sugars, lignin, total soluble phenols, activity of enzymes, and concentration of phenolic compounds and verified the accumulation of phenylpropanoids through histochemical tests in eucalyptus clones that are resistant and susceptible to *C. fimbriata*.

2 Material and Methods

2.1 Plant material, isolate preparation and inoculation

Two hybrid clones (*Eucalyptus grandis* x *E. urophylla*) of eucalyptus were used in this study. One of clones was known to be resistant and another susceptible to wilt caused by *C. fimbriata*. The following treatments were performed: inoculated susceptible clone (SC) and uninoculated (control) susceptible clone (SCC), inoculated resistant clone (RC) and uninoculated (control) resistant clone (RCC). The experiment was conducted in randomized block design composed of four treatments (SC, SCC, RC, and RCC) and four replicates. Each experimental unit (replicates) was composed of three cuttings, totaling 240 plants. The variables analyzed were variations on enzymatic activity as well as on contents of lignin, total phenols and sugars. For sugars, regression models were adjusted by the best model selected based on determination coefficient (R^2) and residue mean square. However, for the phenolic compounds and histochemical studies were carried out in a completely randomized block design composed of four treatments (SC, SCC, RC, and RCC) and six replicates. Each experimental unit (replicates) was composed of one cutting, totaling 24 plants.

In order to certify resistance and conduct the experiments, cuttings plants of 45 days old from plastic tubes were transplanted to pots of 3L capacity containing a mixture of soil, manure and sand (proportion of 4:2:1 v/v/v), fertilized in proportion of 1g of single superphosphate/Kg of mixture. Inoculation was carried out after 30 days of transplanting. The plants were kept in a greenhouse. The isolate RM35, known to be able to cause disease in eucalyptus, was adopted in the experiment (Source: Laboratory of Forest Pathology of Federal University of Viçosa). The isolate was multiplied in a potato-dextrose agar medium (PDA, Himedia®, India) at 28 ºC, photoperiod of 12 h for ten days. After this period, spore suspension was adjusted to 2.5 x 10^6 spores ml^1, according to methodology proposed by Laia et al. (1999) and, immediately inoculated in eucalyptus cuttings. For this purpose, a 2 cm long transversal cut (5 cm above the collecting site) was produced in stem of seedlings. An aliquot of 500 µl of spore suspension of respective isolate was deposited on the injury. The wound was covered with a plastic film to maintain humidity. The plants were maintained in a greenhouse for experiments of enzymatic activity as well as on contents of lignin, total phenols, and sugars. For phenolic compounds experiments and

histochemistry, the plants were kept in a growth chamber (EletroLab®) at 28 °C and 12 hour photoperiod.

2.2 Sample collection and preparation

The analyzed plant material was constituted of longitudinal portions of stem (5 cm above and 5 cm below the injury) of three eucalyptus cuttings. Each sampling unity was composed of three stem sections of three cuttings. Samplings were carried at 2, 4, 7, 14, and 28 days after the inoculation (dai) for the analyses of sugars and activity of defense enzymes such as guaiacol peroxidase (POX), polyphenol oxidase (PPO), and phenylalanine ammonia-lyase (PAL). Aiming to analyze total soluble phenols and lignin, we collected samplings at 7 and 28 dai. For phenolic compounds and histochemical evaluations, we sampled the plant material at 28 dai.

After collection, samples were wrapped in aluminum foil, identified and frozen in liquid nitrogen. Subsequently, we stored the plastic bags in ultra-freezer at -80 °C, until preparation for analyses.

2.3 Quantitative analysis of total soluble sugars

Samples were grinded in ball mill with closed chamber (Marconi® - MA350) for 30 s. Afterwards, potassium phosphate buffer 50 mM pH 7.0 was added (2 ml per each gram of sample). After this procedure, the suspension was centrifuged at 12,000 rpm for 15 min (0 to 4 ^{o}C) and the supernatant was used to estimate sugars. For 20 µl of extract, 500 µl of phenol at 5 % and 2.5 ml of sulfuric acid (PA) were added. The solution was mixed by vortexing and after 15 min of the beginning of reaction and measured in spectrophotometer at 490 nm (Dubois et al. 1956). The results of soluble sugars were expressed in milligrams per gram of dry mass (mg/ g); and the glucose concentrations were used to construct standard curves.

2.4 Determination of soluble lignin and total soluble phenols

Samples collected at 7 and 28 dai were grinded in mill (Marconi®) up to reaching a fine powder. After that, they were freeze-dried for 16 h and used to determine the contents of soluble lignin and total soluble phenols.

2.4.1 Determination of soluble lignin

For soluble lignin content, an aliquot of 30 mg of freeze-dried samples was transferred into a 2ml micro tube, homogenized with 1.5 ml 80% methanol and kept for 15 h, protected from light and at room temperature for tissue depigmentation. Then, the suspension was centrifuged at 12,000 rpm for 5 min, discarding supernatant. The material was wash and 1.5 ml distilled water was added to the solid residue, and subsequently homogenized and centrifuged at 12,000 rpm for 5 min at 4 °C. The supernatant was again discarded and the residue dried in an oven at 65 ºC for 15 h. Posteriorly, we added 1.5ml thioglycolic acid solution (2 N HCl - 1:10). In sequence, the micro tubes were slightly agitated to hydrate the residue and left in water bath at 100 °C for 4 h. Later, the samples were centrifuged at 12,000 rpm for 10 min at 4 °C; the supernatant was discarded and the precipitate washed with 1.5ml distilled water, and once more centrifuged at 12,000 rpm for 10 min at 4 °C. In sequence, the supernatant was disposed and the precipitate was re-suspended in 1.5ml 0.5 M sodium hydroxide and kept in rotary shaker for 15 h at room temperature. The mixture was centrifuged at 12,000 rpm, for 10 min, at 4° C and the supernatant transferred into another micro tube, to which was added 200-µL concentrated HCl. The obtained suspension was

kept under refrigeration (4 ºC) for 4 h, for lignin precipitation with thioglycolic acid. Afterwards, the mixture was centrifuged at 12,000 rpm for 10 min at 4 °C; the supernatant was discarded and the precipitate re-suspended in 2ml 0.5 M NaOH. We determined the absorbance of this solution using a spectrophotometer (BioTek®) at 280 nm and the values calculated based on lignin curve and expressed in µg of soluble lignin per milligram of dry mass (µg/ mg Ms), as described by Doster and Bostock (1988).

2.4.2 Determination of total soluble phenols

For soluble phenols, we transferred aliquots of 30-mg freeze-dried samples into 2ml micro tubes. These samples were homogenized with 1.5ml 80% methanol and remained under agitation for 15 h in a rotary shaker protected from light at room temperature. After this period, the suspension was centrifuged at 12,000 rpm for 5 min. Then, fractions of 150 µL supernatant (methanolic extract) were mixed to 150 µL of 0.25 N Folin-Ciocalteau reagent for 5 min and homogenized with 150 µL 1 M Na_2CO_3 for 10 min and diluted in 1ml distilled water at room temperature for one hour. Absorbance values of this reaction were determined at 725

nm in a spectrophotometer and calculated based on catechol curve. Total phenolic compounds were expressed in equivalent µg of catechol per milligram of dry mass (µg/mg MS) according to the method of Spanos and Wrolstad (1990).

2.5 Sample preparation for evaluation of enzymatic activity

Samples were grinded together with liquid nitrogen in a ball mill with closed chamber (Marconi® - MA350) for 30 s. In sequence, a potassium phosphate buffer (50 mM, pH 7.0) was added in the proportion of 2 ml buffer per sample gram. After this procedure, the suspension was centrifuged at 12,000 rpm for 15 min (0 to 4 ^{o}C); and the resulting supernatant was used as enzymatic source.

2.6 Total Proteins

The soluble protein concentration was estimated with the aid of a standard curve of bovine serum albumin (BSA) according to tests performed by Bradford (1976).

2.7 Enzymatic analyzes

Regarding the enzymes, we evaluated guaiacol peroxidases, polyphenol oxidases and phenylalanine ammonia-lyase. The activity of peroxidases of guaiacol (POX; EC 1.11.1.7) was assessed through the addition of 100 µl enzymatic extract, which was adjusted for 2 ml of a solution containing 900 µl 50 mM sodium acetate at pH 5.2, 500 µl 20 mM guaiacol, and 500-µl 60 mM hydrogen peroxide. After incubation at 30 ^{o}C for 10 min, we measured absorbance using a spectrophotometer (Powerwave, Biotek, Winooski, VT) at 480 nm (Urbanek et al. 1991). A POX unity was expressed as variation of 1.0 OD_{480} per milligram of soluble protein per minute (mg/ P/ min).

The activity of polyphenol oxidases (PPO; EC 1.10.3.2) was determined through the addition of 50 µL enzymatic extract adjusted for 3 ml of a solution containing 100 mM potassium phosphate buffer (pH 6.5) and 25 mM catechol. After 10 min at 30 °C, we quantified the absorbance rise at 410 nm (Gauillard et al. 1993). Results on PPO activity were expressed as change in OD_{410} per milligram of soluble protein within a minute (mg/ P/ min).

Phenylalanine ammonia-lyase activity (PAL, EC: 4.3.1.5) was measured according to method of Mori et al. (2001). For this purpose, we

prepared a reaction mixture by adding 100 µl of 100 mM Tris-HCl buffer (pH 8.8) and 50 µl of 40 mM phenylalanine to 50 µl enzymatic extract. This mixture was incubated for 30 min at 37 °C. The enzyme activity was paralyzed with the addition of 50 µl 6 N HCl. After incubation, we added phenylalanine and HCl to control treatments. The mixture was centrifuged at 1,450 rpm for 15 min at 4 °C. Supernatants were then collected for absorbance readings (180 µl) at 280 nm, evaluating trans-cinnamic acid quantity by comparison with standard curve (0.01–0.10 mg of trans-cinnamic acid ml^{-1}). Results were expressed as unity of activity per milligram of protein per minute (mg/ P/ min).

2.8 Extractions, chromatography and quantification of phenolic compounds

The following Sigma standards were employed for the analyzes: trans-ferulic acid (99%), catechin (98%), chlorogenic acid (95%), ρ-coumaric acid (98%), quercetin, epicatechin (90%), caffeic acid (98%) and acetic acid (HPLC grade) obtained from JT Baker Inc. (Phillipsburg, NJ, USA). Besides the standards before mentioned, we also used acetonitrile (HPLC grade), acetic acid (Merck Chemical, Darmstadt, Germany) and ultrapure water.

Stem samples, with 10 cm, were collected at 28 dai, freeze-dried and ground in a mill (Marconi®) up to reach a fine powder particle size. The extractions were carried out according to methodology adapted from Colaric et al. (2005). In this method, 10ml methanol with 1% 2.6-di-tert-butyl-4-methylphenol (BHT) is added to an aliquot of 150 mg of produced powder. The tubes were kept in ultrasonic bath for 40 min. The extract was centrifuged at 12,000 rpm for 5 min at 10°C, removing the supernatant to a rotary evaporator (Fisatom®) for drying. The dried extract was re-suspended with 2ml methanol and filtered in membrane filters before the injection. The analyzes were carried out in a liquid chromatograph of high efficiency (Shimadzu®, Japan) equipped with quaternary pump LC-20AT model, diode array detector SPDM-20A series, degasser DGU-20A5 model, CBM-20A interface module, and an automatic injector with SIL-20A auto-sampler. Separations were conducted on a 5-µm Shim-pack VP-ODS column (C18, 250 x 4.6 mm - internal diameter) and 5µm Shim-pack GVP-ODS pre column (C18, 10 x 4.6 mm - internal diameter). Elution was conducted in a gradient mode with two solvents (A and B): 0 to 50 min (10-55% B), 50 to 60 min (55-100% B), 60 to 65 min (100-10% B), and 65 to 80 min (10% B). Two

mobile phases were composed of solvent A (water: acetic acid - 98:2% v/v) and solvent B (water: acetic acid: acetonitrile - 49.5: 0.5: 50% v/v). During the entire analysis, we used a flow rate of 1.0 ml min^{-1}. Samples and standards were injected at a volume of 20 µl. All of the samples and standards were analyzed at 280 nm absorbance. The identification of compounds was conducted by comparing sample retention time with the standards. Quantification was carried by external standard concentrations of trans-ferulic acid (0.071 - 1.565 mg l^{-1}), catechin (0.116 – 2.322 mg l^{-1}), chlorogenic acid (0.067 – 1.345 mg l^{-1}), ρ-coumaric acid (0.038 – 0.866 mg l^{-1}), quercetin (0.079 – 1.823 mg l^{-1}), epicatechin (0.061 – 1.582 mg l^{-1}) and caffeic acid (0.061 – 1.582 mg l^{-1}). All samples were analyzed in duplicate and standard working solutions of derived ethyl carbamate in triplicate.

2.9 Histochemistry of stem sections infected with *Ceratocystis fimbriata*

At 28 dai, stem samples were collected and stored at a -80 ºC until preparation for fluorescence test with specific reagents to detect phenolic compounds (Andary et al. 1996).

The plant tissue samples were dampened and sectioned in transversal directions with the aid of a microtome (Leica VT 100M) for histochemical analyses. Then, these sections were contrasted using different reagents:

i) Neu's Reagent: Neu's reagent stock solution (2.5 % w/v) was prepared in 1% ethanol, diluted in 0.1% KPB buffer immediately before use. Stem sections were immersed in the reagent within a small essay tube for approximately 5 min, being lately removed and dipped in distilled water three times consecutively (Hutzler et al. 1998). Sections were arranged on a glass slide containing water-glycerin mixture (1:2 v/v). The slides were then observed in an epifluorescence microscope (Zeiss Observer Z.1) with specific filters for wavelength between 365 nm (excitation) and 445 nm (emission) (4,6-diamidino-2-fenilindole - DAPI).

ii) phloroglucinol-HCl: Lignin was visualized in stem sections using the phloroglucinol reagent dissolved in 96% ethanol for 2 min, washed with 32% HCl and arranged in HCl. After this procedure, compounds with lignin displayed coloration varying between light pink to red (Adler et al. 1948). Tissue sections were arranged on a glass slide with water-glycerin and eventually observed under epifluorescence microscope (Zeiss®) with

specific filters for wavelength between 605 nm (excitation) and 779 nm (emission) (Texas red).

2.10 Statistical analyses

Normality test (Shapiro-Wilk) was conducted for the data obtained. All data had variance analysis (ANOVA) and averages were compared by the Tukey test ($p \leq 0.05$) using the Sisvar 14 software (version 5.3) (Ferreira 2010). P values from analysis of variance of data are shown in Table 1.

Table 1. Analysis of variance of the effects of Clones (C) and time (T), and interactions. P values from analysis of variance of data from a study conducted.

Source of Variation	Degrees of freedom	Soluble sugar	Lignin	Enzyme PPO	Enzyme POX	Enzyme PAL
Clone (C)	3	0.0000	-	-	-	-
Time (T)	4	0.0000	-	-	-	-
C x T	12	0.0162	-	-	-	-
Error	60		-	-	-	-
Total	79					
Clone (C)	3	-	0.0010	-	-	-
Time (T)	1	-	0.0000	-	-	-
C x T	3	-	0.0000	-	-	-
Error	16	-		-	-	-
Total	23					
Clone (C)	3	-	-	0.0000	-	-
Time (T)	4	-	-	0.0000	-	-
C x T	12	-	-	0.0000	-	-
Error	44	-	-		-	-
Total	63					
Clone (C)	3	-	-	-	0.0011	-
Time (T)	4	-	-	-	0.0000	-
C x T	12	-	-	-	0.0000	-
Error	40	-	-	-		-
Total	59					
Clone (C)	3	-	-	-	-	0.1216
Time (T)	4	-	-	-	-	0.0000
C x T	12	-	-	-	-	0.1138
Error	43	-	-	-	-	
Total	62					

3 Results

3.1 Quantitative analyses of soluble sugars

RC plants presented higher quantity of total sugars compared to SC (Figure 1a). Without pathogen inoculation, RC plants also demonstrated higher concentration of sugars when compared to

susceptible clone (Figure 1b). Overall, inoculation promoted changes in sugar content of resistant against susceptible clones. These differences can be observed through the distance between curves. The maximum sugar content for the inoculated RC was of 12.74 mg/g at 22 days, while for SC was of 7.60 mg/g at 21 days (Figure 1a, b).

3.2 Determination of soluble lignin

The quantity of soluble lignin differed between treatments of inoculation and among tested clones. At 7 dai, the lignin contents of susceptible clone (21.36 µg/mg Ms) and of RC (21.69 µg/mg Ms) were lower than the ones from their respective controls, without inoculation. At 28 dai, RC demonstrated higher lignin content (33.22 µg/mg Ms) than SC (29.79 µg/mg Ms), but did not differ from its control (32.55 µg/mg Ms). Overall, lignin content was higher at 28 dai in comparison to 7 dai (Figure 2).

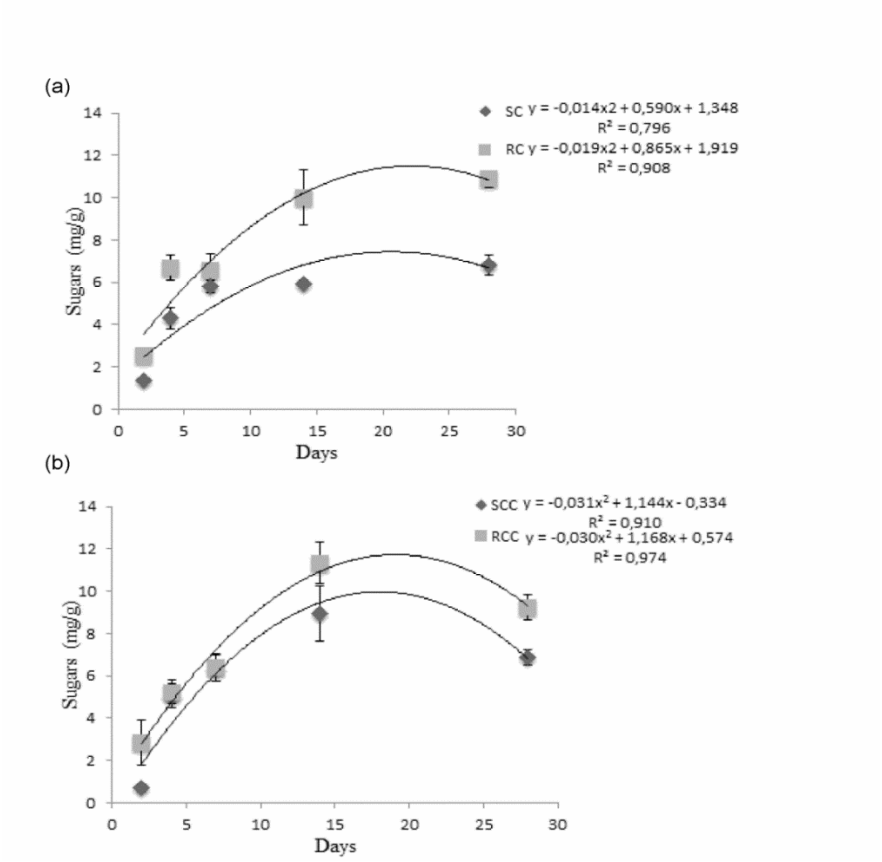

Figure 1 Concentration of soluble sugar (mg/g) in tissue stem *Eucalyptus* clones with different levels of resistance, inoculated and non-inoculated with *Ceratocystis fimbriata* a function of time in days:(a) Clone inoculated susceptible (SC), clone inoculated resistant (RC), (b) clone susceptible uninoculated (SCC) and resistant clone resistant uninoculated (RCC).

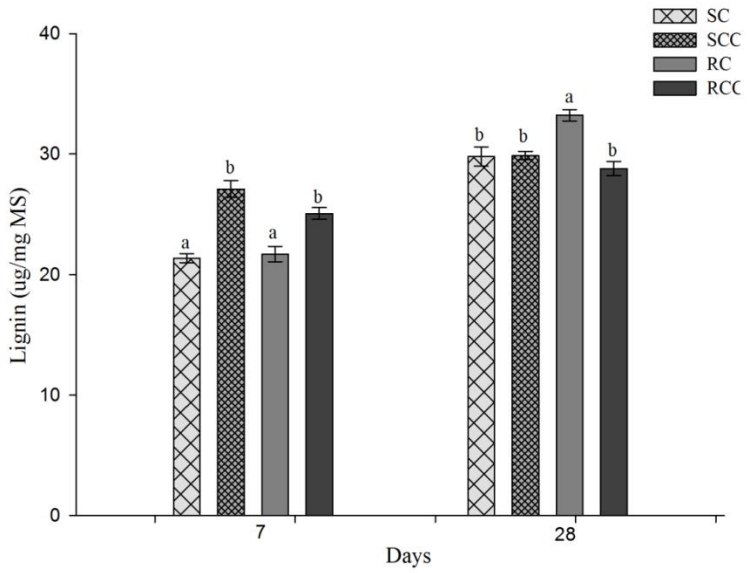

Figura 2 Lignin concentration (mg / mg MS) in stem tissue *Eucalyptus* clones with different level s of resistance, inoculated and non-inoculated with *Ceratocystis fimbriata* a function of time in days. Clone inoculated susceptible (SC), clone inoculated resistant (RC), clone susceptible uninoculated (SCC) and resistant clone resistant uninoculated(RCC). Means followed by the same letter do not differ by Tukey test ($p \leq 0.05$).

3.3 Determination of total soluble phenols

Time and treatment interaction had no effect on total soluble phenols (p= 0.324). Therefore, we analyzed the sources of variation singly. In general, the amount of phenols was higher in all treatments,

except from RCC. In all treatments, the concentration of soluble phenols was superior at 28 dai in comparison to 7 dai**.**

3.4 Enzymatic analyses

The results of enzymatic analyses demonstrated interaction between tested clones and evaluation period for the enzymes PPO and POX. Even though there was no difference among treatments, we observed PPO enzyme activity before 7 days ($p \geq 0.05$) (Figure 3a). At 7 dai, there was a decrease of activity of this enzyme for RC and RCC, while SC and SCC showed an enzymatic activity equivalent to 246 and 200 (mg/ P/ min), respectively. At 14 dai, the enzymatic activity remained active in SC plants (237 mg/ P/ min); however, there was a reduction for the other evaluated treatments. At 28 dai, there was a decrease in activity of enzyme for all the treatments.

POX enzymatic activity in RC and RCC plants was higher at 2 and 4 dai compared to SC and SCC (Figure 3b). As observed for PPO, SC plants started presenting higher activity of POX at 7 and 14 dai. However, there was a decrease in the enzyme production at 28 dai in all treatments.

Concerning the PAL, there was no significant interaction between clones x times (p=0.3404); however, differences regarding time were observed (Figure 3c). Moreover, at 4, 7 and 14 dai, the activity of enzyme in all the treatments was higher compared to the other evaluation periods.

3.5 Determination of phenolic compounds

We observed differences among treatments when assessing compounds referring to hydroxycinnamic acid such as chlorogenic acid, trans-ferulic acid and caffeic acid (p=0.0001). RC and RCC plants presented a quantity of phenolic compounds equivalent to 6.70 mg $L^{-1}$and 3.87 mg L^{-1}, respectively, differing from SC (1.52 mg L^{-1}) and SCC (1.19 mg L^{-1}). Regarding the flavonoids (catechin, quercetin and epicatechin), there were not differences (p=0.0622) among the treatments (Table 2).

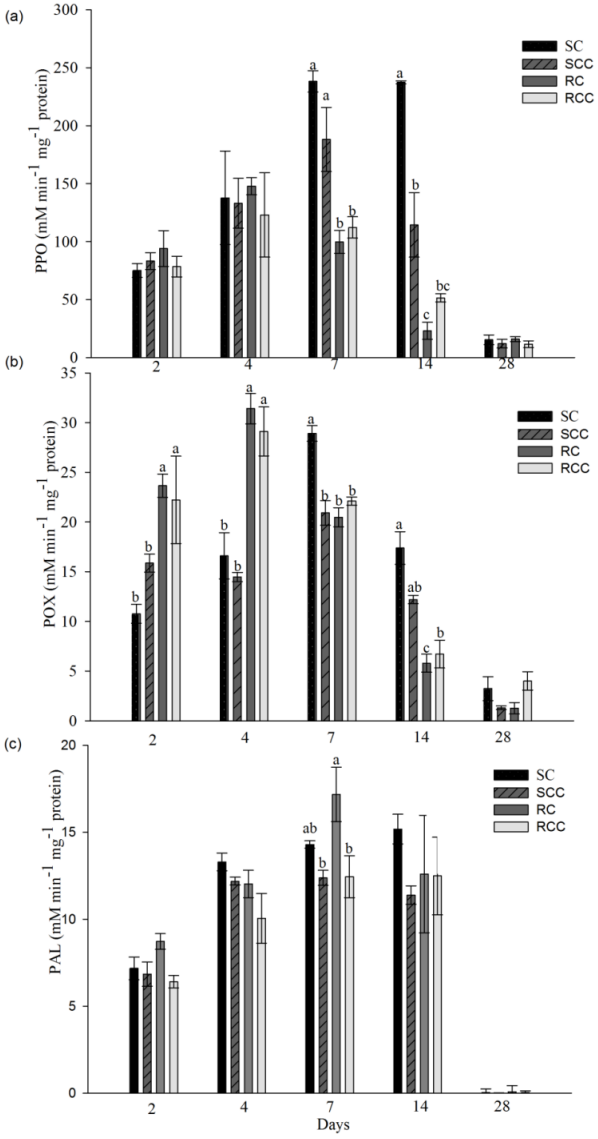

Figure 3 Enzymatic activity in tissue stem *Eucalyptus* clones with different levels of resistance, inoculated and non-inoculated with *Ceratocystis fimbriata* a function of time in days: (a) Polyphenol activity, (b) Guaiacol peroxidase activity and (c) phenylalanine ammonia-lyase activity. Clone inoculated susceptible (SC), clone inoculated resistant (RC), clone susceptible uninoculated (SCC) and resistant clone resistant uninoculated (RCC). Means followed by the same letter do not differ by Tukey test ($p \leq 0.05$).

Additionally, differences were not detected ($p \geq 0.05$) among the treatments with respect to the following compounds: ρ-coumaric acid, quercetin, trans-ferulic acid ($p=0.0679$) and epicatechin ($p=0.1645$). However, some phenolic compounds as catechin, chlorogenic acid and caffeic acid had variance ($p \leq 0.05$) among treatments ($p=0.0264$, $p=0.0148$ and $p=0.0006$ respectively) (Table 2). The catechin concentration in RC plants inoculated with pathogen was of 2.62 mg L^{-1}, differing only from SCC, which presented a concentration of 1.29 mg L^{-1}. Higher amounts of chlorogenic acid in RC plants were observed (1.34 mg L^{-1}) when compared to SC (0.43 mg L^{-1}). The concentration of caffeic acid in RC (3.11 mg L^{-1}) was also significantly superior to SC (1.10 mg L^{-1}).

3.6 Histochemistry of stem sections infected with *Ceratocystis fimbriata*.

The lignin indicator reagent used in the histochemical test showed that RC plants had increased fluorescence intensity compared to SC plants (Figure 4 a, b). The use of inductor reagent of secondary fluorescence in phenolic compounds revealed the same performance,

which means, greater fluorescence in tissues of RC in relation to SC (Figure 4c, d).

Table 2. Concentration hydroxycinnamic acid (chlorogenic acid, caffeic acid, trans-ferulic acid and ρ-coumaric acid) (mg L^{-1}) and flavanols (catechin, epicatechin and quercetin) (mg L^{-1}) detected in eucalyptus clones.

Hydroxycinnamic Acid	**SC***	**SCC***	**RC***	**RCC***	**p***
Chlorogenic acid	0,43 ± 0.05 b	,30 ± 0.013 b	1,34 ± 0.33 a	0,70 ± 0.11 ab	0.0148
Caffeic acid	1,10 ± 0.33 b	,93 ± 0.14 b	3,11 ± 0.30 a	2,49 ± 0.17 a	0.0006
Trans-ferulic acid	0,016 ± 0.05 a	,00 ± 0.001 a	0,012 ± 0.04 a	0,016 ± 0.03 a	0.0679
ρ-coumaric acid	0,00 ± 0.004 a	,00 ± 0.001 a	2,23 ± 0.86 a	0,66 ± 0.65 a	0.0584
Total	1,55 ± 0.13 **c**	,23 ± 0.13 **c**	6,70 ± 0.61 **a**	3,87 ± 0.49 **b**	0.0001
Flavanols					
Catechin	1,53 ± 0.26 ab	,29 ± 0.18 b	2,62 ± 0.36 a	1,97 ±0.16 ab	0.0264
Epicatechin	1,70 ± 0.069 a	,71 ± 0.33 a	2,06 ± 0.58 a	0,85 ± 0.11 a	0.1645
Quercetin	0,21 ± 0.14 a	,24 ± 0.02 a	0,43 ± 0.36 a	0,24 ± 0.12 a	0.8626
Total	3,45 ± 0.42 **a**	,24 ± 1.00 **a**	5,22 ± 1.05 **a**	3,06 ± 0.46 **a**	0.0622

*Inoculated susceptible clone (SC), uninoculated (control) susceptible clone (SCC),inoculated resistant clone (RC) and uninoculated (control) resistant clone (RCC). Means followed by the same letter do not differ by Tukey test ($p \leq 0.05$). Values after ± represent the standard error of the mean.

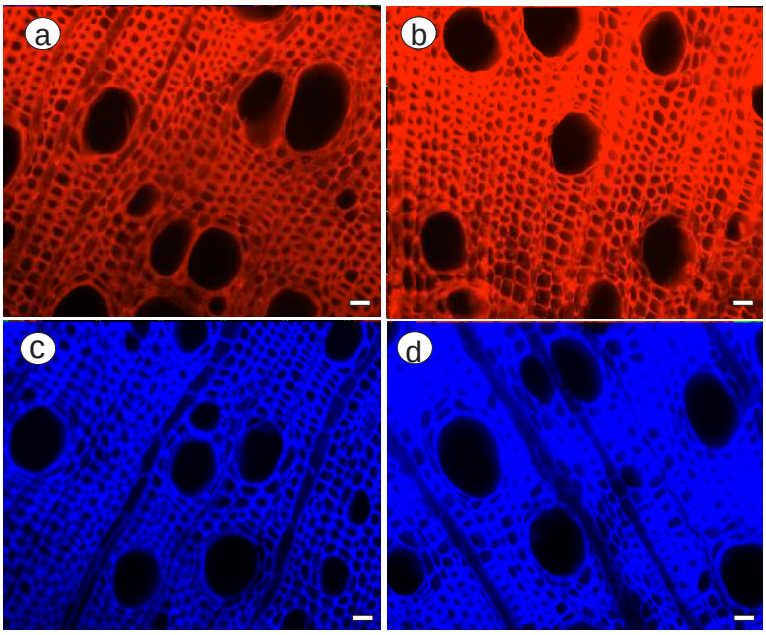

Figure 4 Photomicrograph of epifluorescence. Cross section of susceptible clone (a) and resistant clone (b) inoculated with *C. fimbriata* and stained with phloroglucinol (Filter Set chroma - 39004: Texas red). Cross section of susceptible clone (c) and resistant clone (d) inoculated with *C. fimbriata* and stained with reagent Neus (Filter Set 49: DAPI). Size bar = 20μm.

4 Discussion

In this study, inoculated SC plants showed lower contents of sugars. The sugar consumption by the pathogen is a possible explanation for the difference with uninoculated clones (control). According to Wahl et al. (2010), sugar absorption is an immediate pathogen's strategy to

avoid the induction of plant defense mechanisms. It occurs because these carbohydrate stocks trigger a rapid production or mobilization of cellular defense mechanisms, such as phenolic compounds synthesis, taking place through three metabolic routes (acetate-mevalonate, acetate-malonate and acetate-shikimate) after glycolysis (Resende et al. 2014).

The increased contents of lignin in RC plants denote that the plant might have gonethrough pathogen attack mechanisms, intensifying its lignification process. According to Kúc (2001), cellular wall lignification is characterized as one of the reactions triggered by plant defense system, which aims at preventing penetration or even restricting pathogen colonization of the plant tissues. Bucciarelli et al. (1999) observed that in *Populus tremuloides* resistant to *Entoleuca mammata,* there was lignin accumulation as well as in susceptible genotypes, however, it was more intense in the resistant genotype as we observed here. In addition, El Modafar et al. (2000) observed an increase in lignin content of palm roots for both resistant and susceptible cultivars to *Fusarium oxysporum* f. sp. *albedinis*, being proportionally higher in resistant ones.

Both POX and PPO showed high activity and similar performances. These enzymes have a substantial importance in plant

defense mechanisms (Agrios 2005), once they promote oxidative degradation of phenolic compounds surround the injured areas (Macheix et al. 1986; Bindschedler et al. 2002). In our study, RC plants had initially demonstrated an elevated activity of POX in relation to the SC clones, however, it changed comparatively in relation to SC. The same performance was noticed for the PPO enzyme. This performance can be explained as a delayed reaction of the plant to pathogen invasion in the tissues. It was not observed in RC plants once pathogen was restrained in the early infection stage. Bispo et al. (2015) evaluated changes in the oxidative system of mango cultivars induced by *C. fimbriata* infection. These authors observed an increase of POX activity in the leaves of moderately resistant cultivar (Tommy Atkins) inoculated with the fungus, when compared to the resistant cultivar (Ubá) at 20 and 30 dai. On the other hand, significant differences were not detected for PPO activity. Araújo et al. (2008), evaluating peroxidase activity and concentration of phenols in micro tangerines (*Citrus* spp.) infected by *Phytophthora parasitica*, found that the activity of POX was also higher in susceptible plants by the inoculation of pathogen in plant stems.

In our study, we also found activity of enzymes in tissues of uninoculated clones (control), endorsing that a simple injury on plant tissue is able to stimulate defense responses. Pereira et al. (2008) evaluated defense response in cocoa plants against *Verticillium dahliae* through natural extracts and acibenzolar-S-methyl. In inoculated plants were observed higher activity of POX at 18 dai, inferring that inoculation through injury, by itself, also promotes an increase on the activity of this enzyme.

Regarding PAL, greater activity between 4 and 14 dai was detected in stems of inoculated plants. Bispo et al. (2015) remarked the activity of PAL in plants resistant to *C. fimbriata*, being higher at 30 dai compared to the control. However, these authors evaluated enzymatic activities at tissue areas distant from the inoculation site, e.g. leaves, contrasting from our results, since we evaluated the activity at the infection site. Bispo et al. (2015) also verified that there was no difference of PAL between mango cultivars resistant and moderately resistant. Analyzing the resulting effects of biological activity of PAL enzyme, several studies have highlighted the growth of lignin synthesis in plant tissues (Buchanan et al. 2000; Oliveira et al. 2004), which can represent

an increase of defense ability against pathogens. This is a key enzyme for all synthesis routes of phenolic compounds, which are involved in pest and pathogen resistance. Moreover, the enzyme is responsible for the first of a series of metabolic reactions that generate innumerous natural products based on phenyl propane and including lignin (Pinto et al. 2011).

In this study, the quantity of total soluble phenols at 28 dai was greater than at 7 dai. This difference represents an important aspect as it suggests that greater amounts of phenolic compounds are produced in stems between the two tested periods. Phenols were also registered in cocoa trees resistant to *C. fimbriata* (Capriles de Reys et al. 1964; Capriles de Reys and Reys 1968). Martín et al. (2005) stated that at 30 dai the xylem of *Ulmus minor,* inoculated and colonized by *Ophiostoma*, confirmed a reduction in carbohydrate levels and increase in phenolic compounds, which might have occurred due to the degradation polysaccharides of cellular walls by fungal enzymes as well as the synthesis of defense compounds by the host. The increase of preexisting phenolic compounds represents a response of phenolic metabolism of stress suffered by the plant, like an infection caused by a microorganism. In woody plants, the increased content of flavones, after infection by

several pathogens was already stated and associated with resistance (Treutter and Feucht 1990; Sierotzki and Gessler 1993; Brignolas et al. 1995). Evaluating stems of *P. acerifolia* inoculated with *C. fimbriata* f. sp. *platani*, El Modafar et al. (1996) observed a quick accumulation of flavones around necrotic tissues, suggesting it may avoid parasite installation below and above infection site. Although we have not found differences in flavonoid accumulations, we verified changes in the amounts of hydroxamic acids and their monomeric forms (chlorogenic acid, caffeic acid and trans-ferulic) for the studied clones. Among the hydroxamic acids, chlorogenic acid is one of the most important since when oxidized by polyphenol oxidase enzymes, it originates quinones extremely toxic to microorganisms, besides of playing an essential role in the plant as a metabolic intermediary in the establishment of insoluble compounds associated with resistance (Schwan-Estradas et al. 2008). Capriles de Reys and Reys (1968) carried a biochemical study in cocoa varieties susceptible and resistant to *C. fimbriata*; they detected a major production of phenolic compounds in stems of the resistant variety. The same was observed in this study, in which the quantity of chlorogenic acid was significantly higher in RC in relation to SC.

Through histochemical tests, we observed a positive correlation between stem section coloration intensity and the previously evaluated results. All treatments exhibited coloration in tissues, however, RC demonstrated higher intensity of fluorescence. Likewise, there was a higher concentration of some phenolic compounds and lignin in the resistant clone, when carried the determination of phenolic compounds by HPLC and soluble lignin. Eynck et al. (2009) noticed through histochemical tests that although there was deposition of phenolic compounds and lignin in vascular tissues of resistant and susceptible genotypes of colza to *Verticillium longisporum,* the coloration was most intense in the resistant genotype. Araújo et al. (2015) observed that the isolates of *C. fimbriata* colonized the stem tissues of mango cultivars during infection, however, in resistant cultivars, the majority of infected cells reacted when stained, indicating the presence of phenolic compounds which are formed to act as an antifungal barrier. Kusomoto (2005) investigated the concentrations of lignin and ferulic acid within cellular walls. These authors did not observe any increase in lignin content in necrotic areas of injured stem tissue of *Chamaecyparis obtusa*.

In contrast, concentrations of ferulic acid attached to the cell walls remained high at evaluation time.

Therefore, in this study, we highlighted the importance of some enzymes, mainly involved in defense responses of eucalyptus plants against *C. fimbriata*. In addition, we showed a substantial involvement of phenolic compounds in interactions between *C. fimbriata* and eucalyptus. This is the first study that has assessed the biochemical responses of defense of eucalyptus against infection and colonization by *C. fimbriata*. Thus, further studies are required primarily to elucidate the involvement of additional enzymes apart from PAL, PPO, and POX, in the formation of lignin, phenolic compounds, and other compounds related to plant defense, in eucalyptus resistant clones.

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(VERSÃO PRELIMINAR)

SEGUNDA PARTE

Artigo 2: Phylogeny and cultural characteristics of *Ceratocystis arracacicola* **sp. nov. on** *Arracacia xanthorriza* **in Brazil**

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ABSTRACT

Ceratocystis fimbriata is a complex of many fungal species that has a wide range of hosts, including species of agronomic and forestry importance. Recently, the fungus was observed causing rot and death to Peruvian carrot (*Arracacia xanthorriza).* Therefore, the present work aimed to study the phylogeny of *Ceratocystis* isolates over Peruvian carrot using ITS, β-tubulin, MAT1 and MAT2, as well as interfertility, morphological characteristics and pathogenicity. Phylogenetic analysis of the ITS, MAT1 and MAT2 regions showed that isolates on Peruvian carrot grouped in a clade separate from other *C. fimbriata* isolates, but this was not observed in the β-tubulin region tree, which grouped the isolates on Peruvian carrot along with *C. platani*. The MAT2-male tester strains from Eucalyptus did not successfully cross with MAT1 tester strains of *Ceratocystis* isolates on Peruvian carrot. The result of the crossing was the occurrence of only a few malformed perithecia, with misshapen ostiolar hyphae without ascospore masses on the top of perithecial neck. Regarding the morphology, the isolates on Peruvian carrot showed cylindrical conidia with thicker wall, characteristic not observed in other species of the *C. fimbriata* complex. Pathogenicity tests indicated that the tested isolates were pathogenic to Peruvian carrot, but no symptoms were observed on taro (*Colocasia esculenta*) plants, demonstrating host specificity. The inoculation performed on roots of Peruvian carrot and taro tubers, showed development and production of fungal structures. However, we observed the formation of a reddish halo on taro tubers when inoculated with isolates from Peruvian carrot,

showing that host response may be present and blocking the pathogen attack. Based on the phylogenetic evidence, mating experiment results, morphology and host specialization, a new species associated to Peruvian carrot was herein described as *Ceratocystis arracacicola* sp. nov.

Keywords: Root rot, Peruvian carrot, Ceratocystis wilt, Phylogeny

INTRODUCTION

The Peruvian carrot (*Arracacia xanthorrhiza* Bancroft) is a typical South American plant of the Andes. According to Balbino et al. (1990), this plant was introduced in Brazil at the beginning of last century and since then its cultivation spread to the states of the Center and Southern region. Brazil is amongst the major producers of this crop in South America with a planted area of 16.603 ha and annual average production of 154.485 tons in these areas (Madeira and Souza 2004).

Despite this vegetable be considered a rustic plant with good tolerance to many diseases and pests, and showing rare reports of severe losses, the Peruvian carrot is a highly exposed crop because of the relatively long crop cycle (8-11 months), which exposes the crop to favorable conditions of disease development, especially during the hot

and rainy seasons (Henz et al. 2008). Compared with other vegetables, the number of pathogens recorded on Peruvian carrot can be considered low and of secondary importance (Henz 2002). However, recently it was observed the presence *Ceratocystis fimbriata* Ell & Halst causing black rot and death on Peruvian carrot plants, affecting significantly the crop yield in Poço Fundo, Minas Gerais, Brazil (Melo et al. 2015). Besides the Peruvian carrot, the fungus was reported causing black rot on other hosts of agronomic importance, such as taro (*Colocasia esculenta*) (Harringnton et al. 2005) and sweet potato (*Ipomoea batatas*) (Halsted and Fairchild 1890).

In the last years, it has been clarified that isolates of *C. fimbriata* of different hosts and different geographic locations may differ in several characteristics (type of colony, pathogenicity, growth rate, and spores) emphasizing the importance of full study for taxonomy of *C. fimbriata*. Using microsatellite markers associated with interfertility experiments, the study of Ferreira et al. (2010) showed that Brazilian isolates of *C. fimbriata* from many different hosts belong to a single biological species within the Latin American clade. Subsequently, Harrington et al. (2014) have assessed the intraspecific and intragenomic variability of ITS rDNA

sequences of some recently described species of *C. fimbriata* complex, and it became clear that many of those species were considered as new based solely on variation of ITS sequences (Johnson et al. 2005; Harrington et al. 2011; Thorpe et al. 2005; Van Wyk et al. 2007, 2009, 2010, 2011a, 2011b, 2012) and they proposed that description of new species in this group of fungi should be conducted using other characteristics than ITS sequences alone. Additionally, Oliveira et al. (2015) compared populations of *C. fimbriata* on mango from Oman, Pakistan and Brazil and showed that recently named species (*C. manginecans*, *C. acaciivora*, *C. mangicola*, and *C. mangivora*) are considered synonymous of *C. fimbriata*.

Black rot was recently reported on Peruvian carrot, and the causal agent was attributed to *C. fimbriata* (Melo et al. 2015). However, more detailed studies are necessary to elucidate the taxonomy of *Ceratocystis* isolates on Peruvian carrot (Harrington et al. 2014; Oliveira et al. 2015). Therefore, this study aimed to determine the phylogenetic relationships, sexual compatibility, morphological characteristics, and pathogenicity of *Ceratocystis* isolates obtained from diseased plants of *A. xanthorriza* and compares them with isolates of taro.

MATERIALS AND METHODS

Collection of isolates

The characterization of disease symptoms was based on observations of roots of Peruvian carrot in Poço Fundo, Minas Gerais, Brazil. Samples of infected roots and propagation material (corms) were collected from plants with symptoms of black rot and stored in paper bags. The samples were processed at the Laboratory of Forest Pathology at the Federal University of Lavras (UFLA). The fungus was isolated from the roots and propagation material tissues by placing the diseased portion in baits of sandwich carrots and incubated at 25 °C (Moller and DeVay 1968). Seven days later, ascospores masses formed at the apex of perithecia were transferred to MYEA media (2% malt extract, 0.2% yeast extract, and 2% agar). Pure cultures were stored on glycerol 15% at -80°C in the Forest Pathology Laboratory, UFLA.

DNA sequencing and phylogenetic analysis

The isolates were grown on MYEA and incubated at 28 °C for 7 days and the mycelium was scraped off, macerated in liquid nitrogen and DNA extraction was Performed using Wizard®Genomic DNA purification kit (Promega®, Brazil), with some modifications: initially the mycelium was

transferred to 2 mL tube containing 200 μL of nuclei lysis solution, the mycelium was homogenized in an extractor (Tissuelyser III, Qiagen) at 30Hz for 2 min, incubated at −20 °C for 5 min, 400 μL of nuclei lysis solution was added, again macerated, and incubated 65 °C for 15 min (tubes inverted every 5 min). After centrifugation for 5 min at 13,000 g, the supernatant was incubated for 5 min at room temperature, 300 μL of protein precipitation solution was added and centrifuged at 13,000 g for 10 min. A mixture of 500 μL of the supernatant and 500 μL of phenol:chloroform:isoamyl alcohol (25:24:1) was stirred and centrifuged at 13,000 g for 5 min, and the aqueous phase was added to 600 μL of cold isopropanol. After 12 h on −20 °C and centrifugation (7 min at 13,000 g), the supernatant was discarded and the pellet washed three times with 600 μL of cold ethanol (70 %). The resulting pellet was dried at room temperature, resuspended in 50 μL of DNA rehydration solution plus l μL of RNase solution, and incubated at 37 °C overnight, followed by 65 °C for 10 min. The concentration of purified DNA was quantified with a Nanodrop 2000c (Thermo) and adjusted to 50 ng/μL.

For amplification and sequencing of ITS (internal transcribed spacer) region we used the primers ITS1 (5′-

CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3) and the following cycling conditions: 85 °C for 2 min, 95 °C for 95 sec, and then 36 cycles of 58 °C for 1 min, 72 °C for 80 sec and 95 °C for 70 sec, followed by a 52 °C for 1 min and 72 °C for 15 min (Harrington et al., 2011). The amplified fragments were purified using the GenElute PCR Clean-up Kit (Sigma-Aldrich®).

All isolates used in the present study have genes associated with both the MAT2 and MAT1 mating types in the mating type locus. The primers CFMAT1-F (5' –CAGCCTCGATTGAKGGTATGA-3') and CFMAT1-R (5' –GGCATTTTTACGCTGGTTAG-3') were used to amplify and sequence about 1000 bp region of MAT1-1-2. The primers X9978a (5' - GCTAACCTTCACGCCAATTTTGCC- 3') and CFM2-1F (5' AGTTACAAGTGTTCCCAAAAG- 3') were used to amplify and sequence about 1150 bp that included most of the region MAT1-2-1. The thermocycler settings for amplifying the MAT1 and MAT2 regions included: initial denaturation at 94 °C for 2 min, with 36 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 2 min, and a final extension of at 72 °C for 10 min. The amplified products were sequenced with the PCR primers.

For sequencing β-tubulin (BT) region, the primers BT1-α (5' - TTCCCCCGTCTCCACTTCTTCATG - 3') and BT1-β (5' - GACGAGATCGTTCATGTTGAACTC - 3') (Glass and Donaldson, 1995) were used to amplify and sequence about 600 bp and thermocycler settings for amplifying the β-tubulin region included: initial denaturation at 94 °C for 4 min, with 30 cycles of 94 °C for 50 sec, 55 °C for 50 sec, 72 °C for 50 sec, and a final extension of at 72 °C for 10 min.

Amplification and sequencing was performed in Forest Pathology Laboratory of the Federal University of Viçosa. The electropherograms were generated using the edited program SeqAssem (Hepperle 2004). Multiple alignments of nucleotide sequences were generated using the tool CLUSTALW (Thompson et al. 1992) implemented by the MEGA 6.0 program and were corrected manually, when necessary. Phylogenetic trees were generated in PAUP software 4,0b10 * (Swofford 2002) and MrBayes v. 3.2.1 (Ronquist et al. 2003) using methods Maximum parsimony and Bayesian inference. Phylogenetic trees were viewed and edited in FigureTree 1.3.1. (http://tree.bio.ac.uk/software). Sequences of *C. variospora* were defined as outgroup taxon.

Morphological characterization

One isolate of Peruvian carrot (LP9) was used for morphological studies. Cultures were grown on MYEA and incubated at 28 °C for about 7 days with a photoperiod of 12 hours. Measurements were made after 10 days. Fungal structures were mounted into lactic acid and observed in light microscope (Leica®). Conidia, ostiolar hyphae and aleuroconidia were measured at 400× magnification, conidiophores were measured at 200× magnification and perithecia with 100× magnification. Measurements were taken from 30 representative spores or structures.

Mating experiments

As determined in previous studies (Engelbrecht and Harrington 2005; Ferreira *et al*. 2010; Johnson et al. 2005; Oliveira et al. 2015), sexual compatibility (interfertility) was determined in the present study following the protocols used by the referenced authors. The most studied Peruvian carrot isolate (LP9) was selected as female tester, along with isolates on mango (SESP8-3), andiroba (RR74), rubber tree (A05), teak (MT29), eucalyptus (SBS1), and cacao (C1587), and crossed with a male colony (mutant) of *C. fimbriata* on *Eucalyptus* spp. (isolate SBS1, from Forest Pathology Lab. of Federal University of Viçosa) in mating experiments. Five plates of the female testers were used as recipient and

grown on MYEA for 10 days at room temperature (approximately 23 °C) and then spermatized by male testers (SBS1). The colonies were observed for 3-4 weeks for the presence of perithecia and ascospore masses. The presence of abundant, normal-appearing ascospores indicated tha a good cross occurred. In a presence of no ascospores or occurrence of only a few misshapen ascospores or occurrence of ostiolar hyphae malformed, we considered as an interspecific cross or hybrid (Ferreira et al. 2010; Harrington and McNew 1998; Johnson et al. 2005; Oliveira et al. 2015).

Pathogenicity tests

Cross inoculation experiments with isolates of Peruvian carrot and taro (*C. esculenta*) were used in pathogenicity tests. We used one isolate from Peruvian carrot (LP9) from this study and one isolate from taro (C1905). The isolates were grown on MYEA and incubated at 28 °C for about 7 days with a photoperiod of 12 hours. After this period, a spore suspension was adjusted (2.5×10^6 spores / ml) for inoculation in plants of Peruvian carrot and taro with 40 and 60 days of age respectively, and grown in vases with 3 kg capacity containing soil, muck and sand (ratio 4: 2: 1 v / v / v). A cross-section of approximately 2 cm length was made on collar of plants (interface root and aerial part).The amount of 500 µl of spore

suspension was deposited over the wound and covered with plastic wrap to maintain humidity of inoculum. The control plants were inoculated with distilled water and submitted to the same conditions. The plants were maintained in a greenhouse for 28 days during the month of June 2015 and after this period the lesion size was measured. The experiment was conducted in randomized block design composed of two isolates and two hosts, each with four replicates.

At the same time, pathogenicity tests were conducted using roots of Peruvian carrot and tubers of taro, which were inoculated with the same two isolates used in previous experiments (LP9 and C1905). The isolates were inoculated on the roots by depositing 500 µl of spore suspension adjusted to 2.5×10^6 spores / ml. The roots were maintained at 25 ° C, photoperiod of 12 h for seven days, and after that the lesion size was measured. The control was inoculated with distilled water and submitted to the same conditions. The experiment was conducted in completely randomized design composed of four treatments with four replicates.

Normality test (Shapiro-Wilk) was conducted for the data obtained from both pathogenicity experiments: seedlings and roots. When did not

meet the assumptions of normality, the data were transformed into Log (x + 1). Data were submitted to analysis of variance (ANOVA) and the means of each treatment were compared by the Tukey test ($p \leq 0.05$) using the Sisvar 14 software (version 5.3) (Ferreira 2010).

RESULTS

Collection of isolates

Thirty samples were collected in the area where the disease occurred. Ten isolates obtained from the field, five were removed from the propagating material and five were taken from the roots. The isolates were all obtained from a single plantation of Peruvian carrot in Poço Fundo, Minas Gerais, Brazil. Plants of Peruvian carrot were observed in the field showing yellowing symptoms in the leaves, followed by drying and death of the plant. Black rot was observed in the roots as well as in propagation material (Figure 7), from where we detected the presence of perithecia, typical of species of the *Ceratocystis fimbriata* complex.

Phylogeny

Trees were generated for each region studied. Initially, a phylogenetic tree was generated for the ITS region, only for Maximum Parsimony

method, using sequences of 10 isolates of Peruvian carrot from this study along with 63 sequences of *Ceratocystis* isolates belonging Latin America, North America, Africa and Asia clades. The results showed the grouping of Peruvian carrot isolates in Latin American Clade (Figure 1). This analysis generated a total of 100 trees of 470 steps. The total number of characters was 620, with 379 constant, 19 parsimony non-informatives and 222 parsimony informatives. The branch with Peruvian carrot isolates was supported with a bootstrap value of 72%.

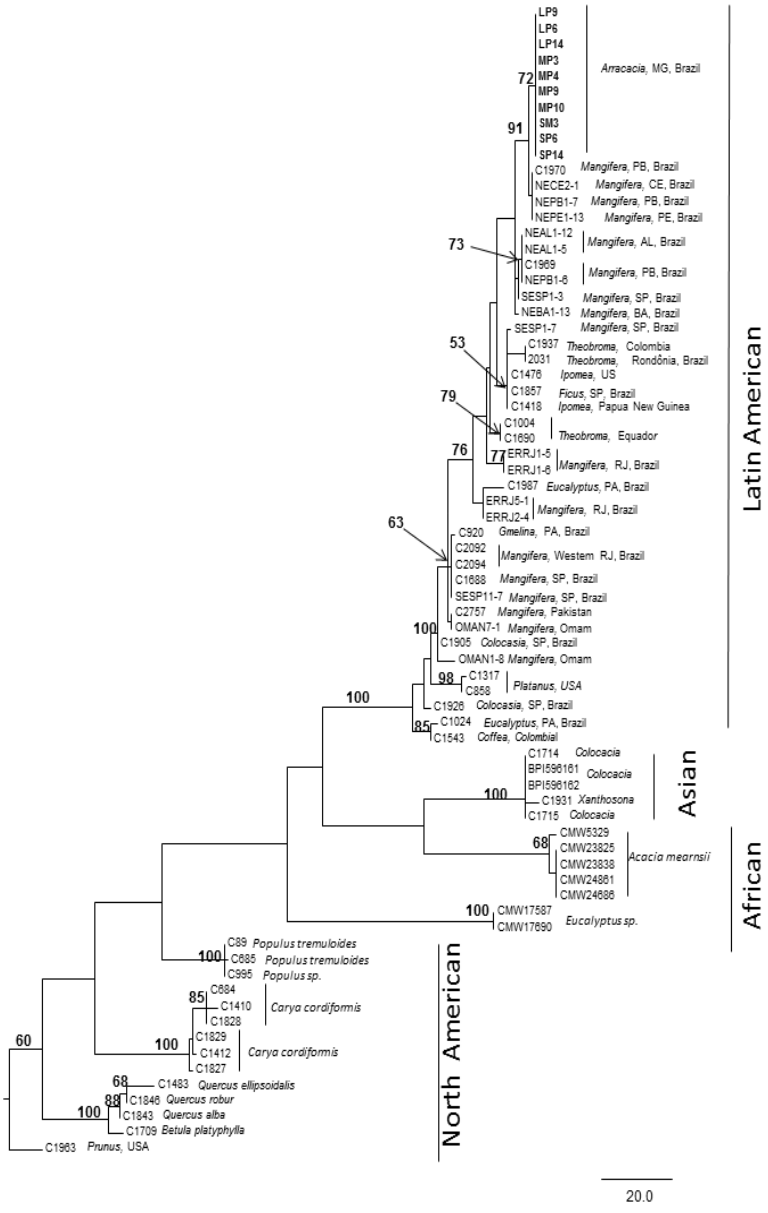

Figure 1 One of 100 trees of Maximum Parsimony of 470 steps using 620 characters of *Ceratocystis arracacicola* isolates from ITS region (in bold) and others belonging to isolated clades of Latin America, Asia, Africa and North America. The *Bootstrap* values are shown to the right. Scale bar indicates number of base changes.

Ten ITS sequences of Peruvian carrot isolates were compared with 58 *Ceratocystis* sequences belonging to the Latin America clade and using *C. variospora* as an outgroup. With the alignment of 604 bp, a total of 2441 trees were generated by 134 steps. The total numbers of characters were 515, with 40 parsimony non-informatives and 49 parsimony informatives. The Peruvian carrot sequences grouped into a separate and unique branch and it was supported by high bootstrap value (97%) and high posterior probability value (0.91) (Figure 2).

As conducted for ITS region, the same ten isolates were sequenced for BT region and compared with 58 sequences of isolates of *Ceratocystis* species the Latin America clade. One hundred trees were generated by 49 steps. The total number of characters was 547, with 500 constant, 33 parsimony non-informatives 14 parsimony informatives. The Peruvian carrot isolates had moderate to high values of bootstrap and posterior probability (73% and 0.97 respectively). Unlike the ITS tree, sequences of BT region clustered the Peruvian carrot isolates with isolates of *C. platani* (Figure 3).

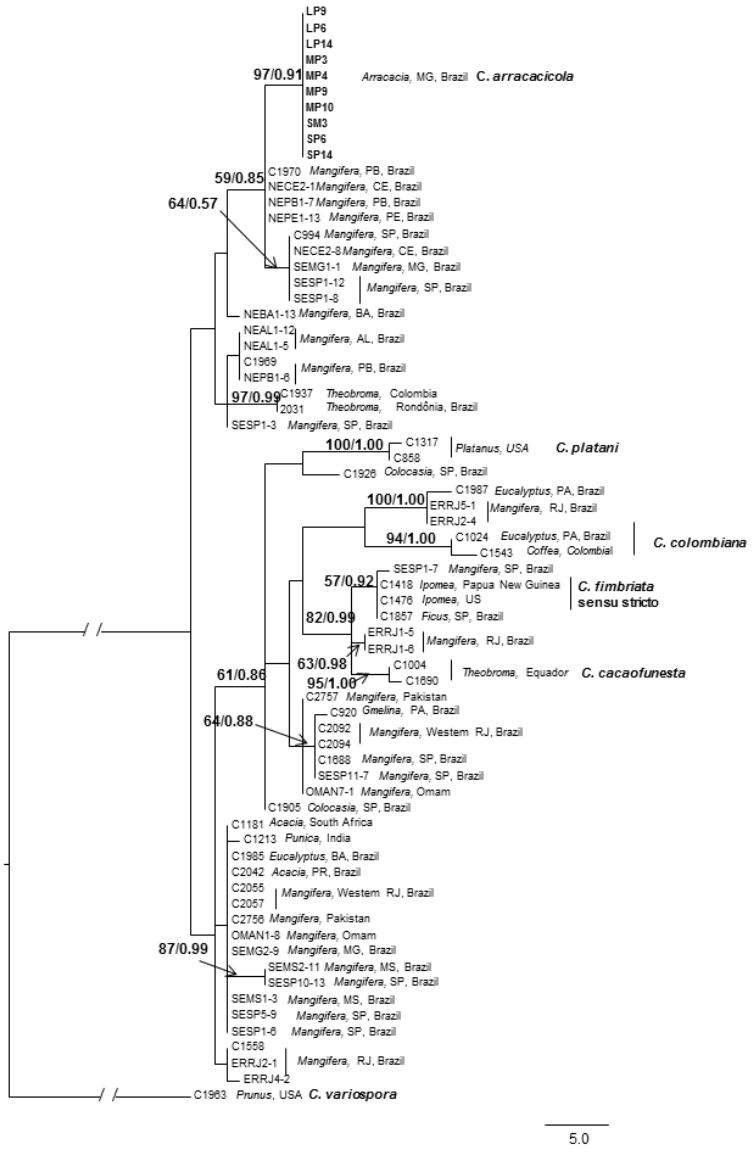

Figure 2 One of 2441 trees of Maximum Parsimony of 134 steps using 515 characters of the ITS region of isolates of *Ceratocystis arracacicola* (in bold) and other isolates of *Ceratocystis*. The *bootstrap* values (>50%) and posterior probability of the clades are shown the right and left, respectively, in the branches. Isolates numbers are followed by host genus and country. *Ceratocystis variospora* the North American clade was defined as *outgroup*. Scale bar indicates number of base changes.

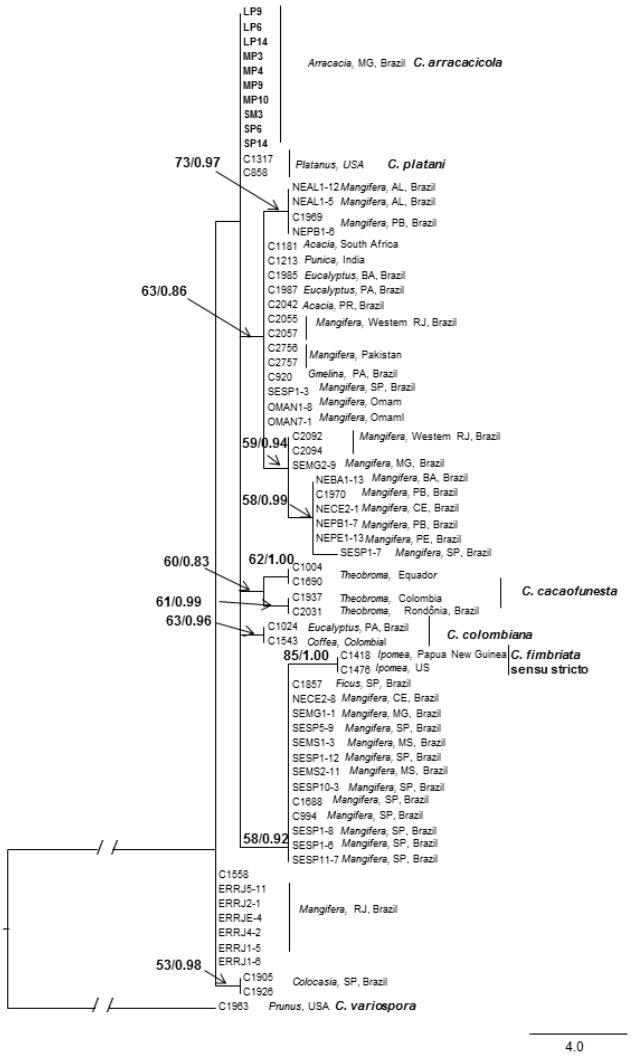

Figure 3 One of 100 trees of Maximum Parsimony of 49 steps using 547 characters of the β-tubulina region of isolates of *Ceratocystis arracacicola* (in bold) and other isolates of *Ceratocystis*. The *bootstrap* values (>50%) and posterior probability of the clades are shown the right and left, respectively, in the branches. Isolates numbers are followed by host genus and country(abbreviation for Brazilian state, where enclosed). *Ceratocystis variospora* the North American clade was defined as *outgroup*. Scale bar indicates number of base changes.

For MAT1 region, a total of 24 trees were generated of 167 steps. The dataset contained 69 sequences with a total alignment of 1030 characters, with 868 constant, 137 parsimony non-informatives and 25 parsimony informatives. Peruvian carrot isolates grouped into a single clade, which was supported by high bootstrap values (93%) and high posterior probability (1.0) (Figure 4). For MAT2, three trees were generated of 308 steps. The dataset sequences had an alignment of 1119 characters, with 837 constant, 219 parsimony non-informatives and 63 parsimony informatives. As observed for MAT1, the bootstrap values and posterior probability for MAT2 region were very high (100% and 0.98 respectively) and Peruvian carrot isolates were grouped in a separate clade (Figure 5).

The Partition Homogeneity Test (PHT) allowed to combined all studied regions (P = 0.01), even with a low support (Cunningham, 1997). For Maximum Parsimony analysis, a total of 245 trees were generated of 757 steps. The dataset analyzed had an alignment of 3330 characters, with 2720 constant, 429 parsimony non-informatives and 151 parsimony informatives. The topology of the concatenated tree showed clearly the grouping of Peruvian carrots isolates in a single clade, with high bootstrap

support and high posterior probability (100% and 0.99 respectively) (Figure 6).

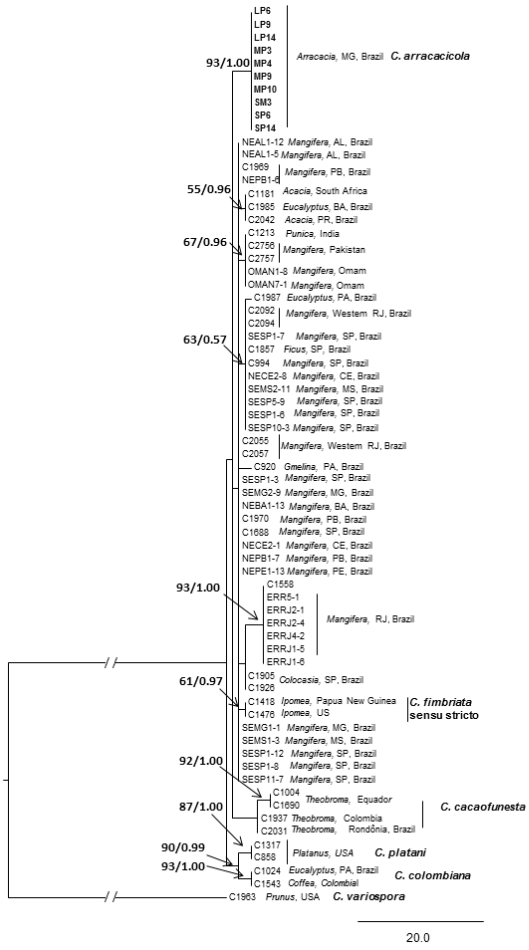

Figure 4 One of 24 trees of Maximum Parsimony of 167 steps using 1030 characters of the *MAT1-1-2* region of isolates of *Ceratocystis arracacicola* (in bold) and other isolates of *Ceratocystis*. The *bootstrap* values (>50%) and posterior probability of the clades are shown the right and left, respectively, in the branches. Isolates numbers are followed by host genus and country (abbreviation for Brazilian state, where enclosed). *Ceratocystis variospora* the North American clade was defined as *outgroup*. Scale bar indicates number of base changes.

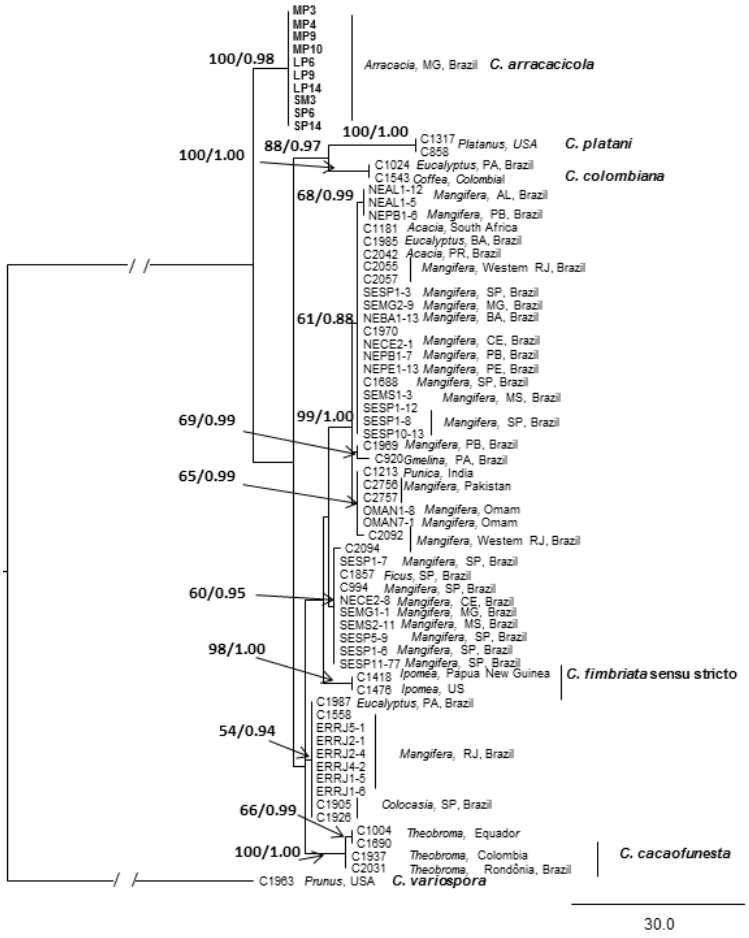

Figura 5 One of three trees of Maximum Parsimony of 308 steps using 1119 characters of the *MAT1-2-1* region of isolates of *Ceratocystis arracacicola* (in bold) and other isolates of *Ceratocystis*. The *bootstrap* values (>50%) and posterior probability of the clades are shown the right and left, respectively, in the branches. Isolates numbers are followed by host genus and country (abbreviation for Brazilian state, where enclosed). *Ceratocystis variospora* the North American clade was defined as *outgroup*. Scale bar indicates number of base changes.

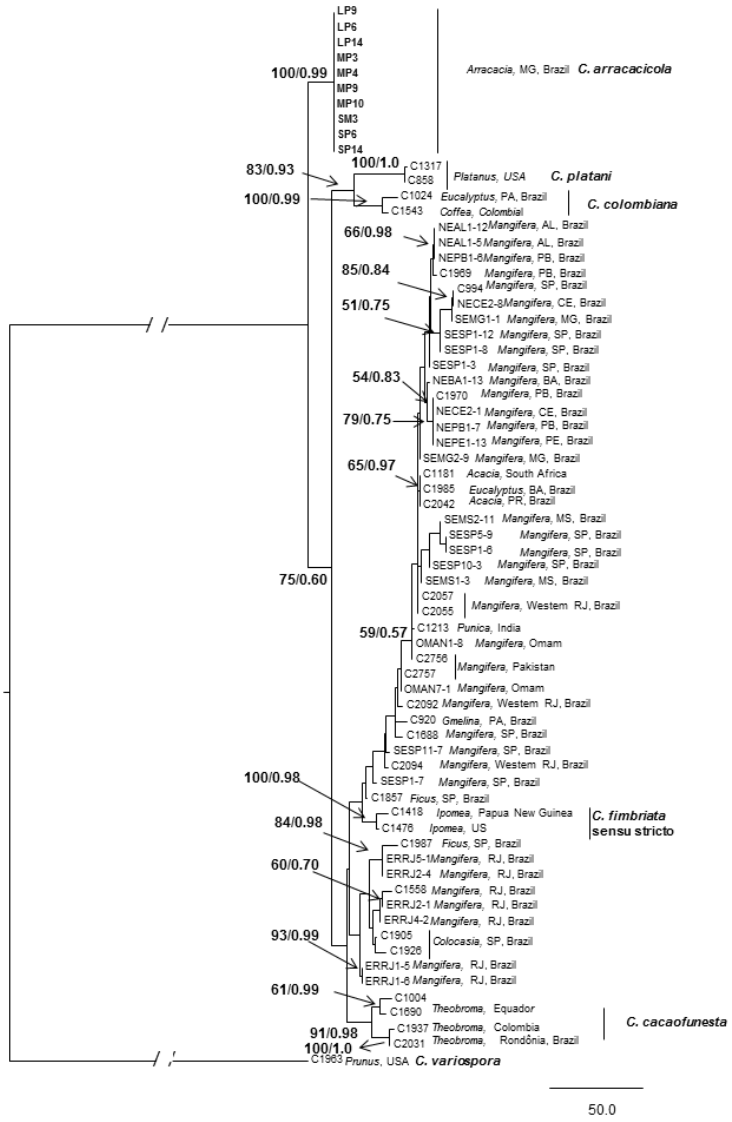

Figure 6 One of 245 trees of Maximum Parsimony of Tree of 167 steps 1030 using characters of consensus regions combined ITS, β-tubulina, *MAT1-1-2* and *MAT1-2-1* of strains of *Ceratocystis arracacicola* (in bold) and other isolates Ceratocystis. The *bootstrap* values (>50%) and posterior probability of the clades are shown the right and left, respectively, in the branches. Isolates numbers are followed by host genus and country (abbreviation for Brazilian state, where enclosed). *Ceratocystis variospora* the North American clade was defined as *outgroup*. Scale bar indicates number of base changes.

Mating experiments

Fully-developed perithecia were observed in successful, interfertile (I) cross, that were identified within a week. Creamy ascospore masses on the top of each perithecial neck were the sign of a successful cross (Table 1, Figure 7). Ascospore masses were transferred to plates containing MYEA, and individual colonies from fully interfertile crosses produced colonies of the mycelial morphology of both the male and female tester strains, demonstrating that the ascospore masses were not due to a selfing (Ferreira et al., 2010; Oliveira et al., 2015).

The MAT2-male tester strains from *Eucalyptus* did not cross successfully with MAT1 tester strains of *C. cacaofunesta* and *C. arracacicola* (Table 1). In the interspecific cross with the isolate of *C. cacaofunesta*, only a few perithecia were produced and the ascospore masses from these perithecia were watery, not creamy. Crosses with male tester isolate of *C. fimbriata* and the female tester isolate on Peruvian carrot showed occurrence of only a few malformed perithecia, with misshapen ostiolar hyphae without ascospore masses on the top of perithecial neck, which were observed by microscopic examination (Figure 8), suggesting a post-zygotic barrier to meiosis.

Table 1 - Interfertiliy (I) and interspecific hybrid (H) interactions between MAT2, male-only strains of *C. fimbriata* from *Eucalyptus* with MAT1, female strains of *Ceratocystis fimbriata*, *Ceratocystis cacaofunesta* and *Ceratocystis arracacicola*.

Species	Host	MAT1, Female[a]	MAT2, Male[a]
			SBS1sec
C. fimbriata	*Eucalyptus* sp.	SBS1ss	I[b]
	Mangifera indica	SESP8-3ss	I
	Carapa guianensis	RR74ss	I
	Hevea brasiliensies	A05ss	I
	Tectona grandis	MT29ss	I
C. cacaofunesta	*Theobroma cacao*	C1587ss	H[c]
C. arracacicola	*Arracacia xanthorrhiza*	LP9ss	H

[a]sec = strains from MAT2, self-sterile sectors recovered from self-fertile isolates; ss = MAT1, female-competent isolates with protoperithecia.

[b]I = Interfertile: ascospores abundant, with normal form; exuded ascospore masses creamy. Presence of both parental types among progeny when ascospores mass transferred and spread across a new plate.

[c]H = Hybrid: much cellular debris and few misshapen ascospores inside perithecium; exuded ascospore masses, when present, watery in appearance; ostiolar hyphae malformed.

Figure 7 Successful cross between two compatible strains of *Certocystis fimbriata* (isolates SBS1sec on *Eucalyptus* and SESP8-3ss on *Mangifera indica*). **a**, Colony; **b**, Ostiolar hyphae; **c**, Ascospores; **d**, Perithecia.

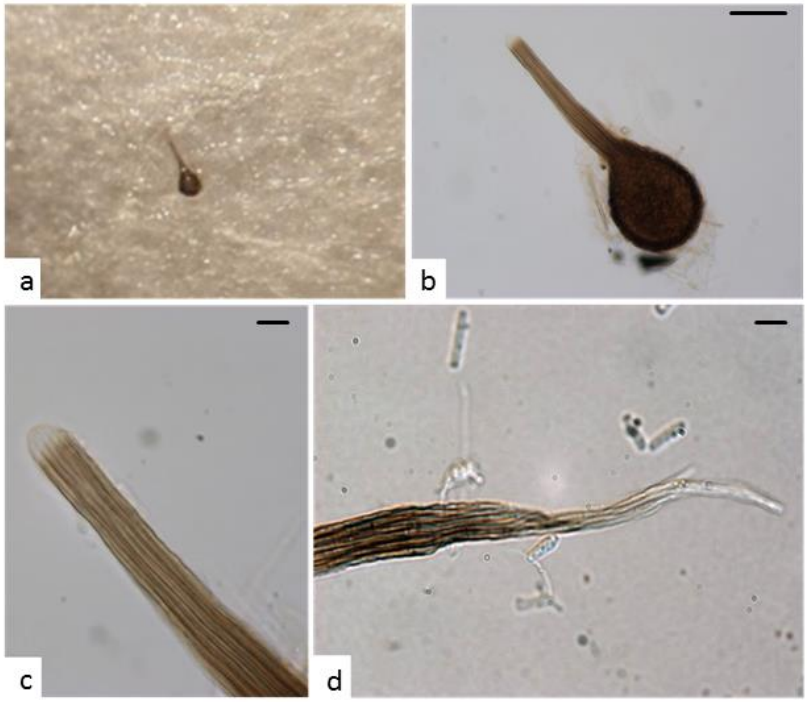

Figure 8 Hybrid cross between an *Eucalyptus* strain (SBS1sec) of *Certocystis fimbriata* and a Peruvian carrot strain (LP9ss) of *C. arracacicola*. **a**, Colony; **b**, Perithecia; **c** and **d**, Ostiolar hyphae.

Taxonomy

Ceratocystis arracacicola, L. Pimenta, M.A. Ferreira & L.S.S. Oliveira. sp. nov. **(**Figure 9a-e)

MycoBank XXXXXX

Holotype BPI: CML 3304 deposited on Mycological Collection of the Federal University of Lavras).

Etymology: *arracacicola:* The epithet refers to the occurrence on *Arracacia.*

Description. Colonies on MYEA showing radial mycelial growth, with rates varying from to 4 – 6,5 cm after 7 days at 28°C.Colony color on MYEA is dark gray (Figure 9a). *Perithecia* of isolates on Peruvian carrot were found superficial or embedded in substrate, coloration dark brown to black, globose, with base measuring 105-180 µm x 103-179 µm. *Neck* dark brown to black were long and exhibited the same color as the base, 269-643 µm, 18-28 µm at the base and 12-17 µm width at the apex (Figure 9c). *Ostiolar hyphae* are light brown to hyaline, non-septate, 23-47 µm long (Figure 9f). Asci not seen. *Ascospores* hat shaped, hyaline, 1.9 - 3.5 x 3.1 - 4.6 µm (Figure 9e). The *endoconiophore* producing barrel conidia are hyaline or pale brown, septate, 2.2 - 4.1 µm width at the base, 2-5 µm width at the widest point, and 33.6 - 159 µm length, producing chains of cylindrical endoconidia. The other endoconidiophore was less common, shorter, producing chains of doliform endoconidia (barrel-shaped) (Figure 9d). *Barrel-shaped* endoconidia were also hyaline, 13.9 - 19.4 µm x 6.5 - 8.4 µm. *Cylindrical endoconidia* were hyaline, 6.4-18.6 µm x 2 - 4 µm with some presenting the thicker wall in the ends (Figure

9g). The *aleurioconidia* were brown, ovoid to pear-shaped, produced singly or in chains, 8.3 - 13.9 x 6.8 - 11.2 µm (Figure 9b).

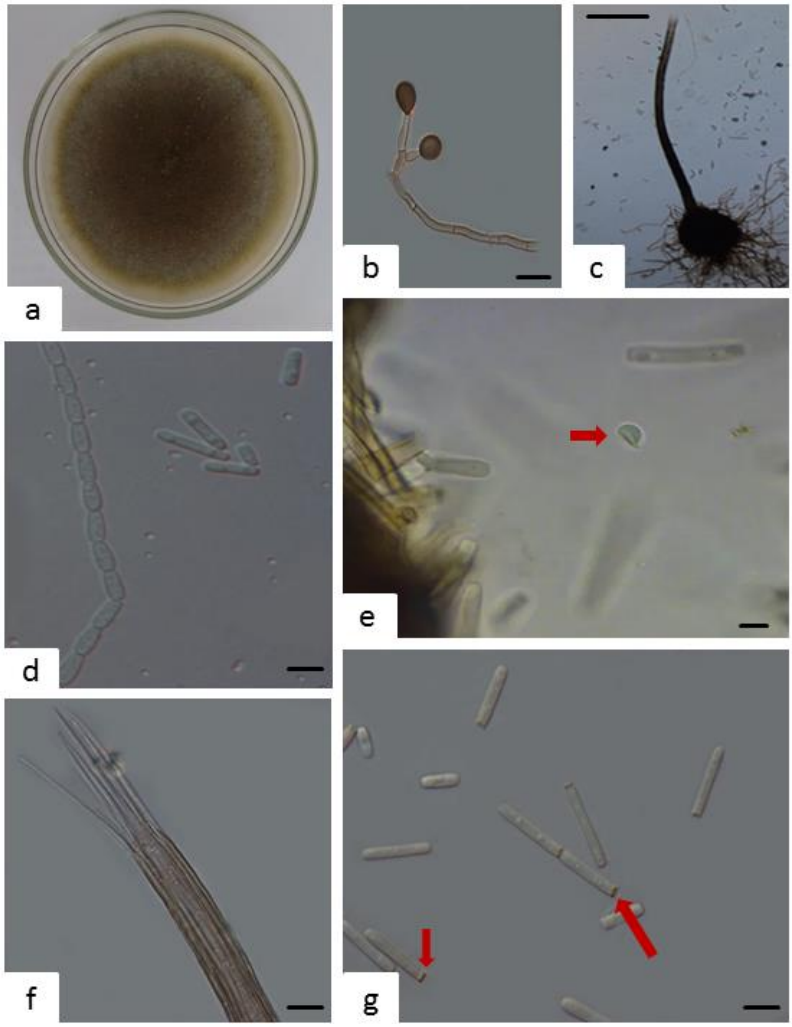

Figure 9 Morphological characteristics of *Ceratocystis arracacicola*. **a**, Colony on MYEA; **b**, Aleuroconídios, Bar =10 µm; **c**, Perithecium, Bar =100 µm; **d**, Conidia, Bar =20 µm; **e**, Ascospore-shaped hat (Arrow indicates), Bar =5 µm; **f**, Hyphae ostiolares, Bar =20 µm; **g**, Conidia cylindrical (Arrow indicates wall thickening), Bar =20 µm.

Pathogenicity tests

In the pathogenicity test using seedlings, there was no interaction between plant × isolates (P = 0.156). The peruvian isolate was not able to cause black rot on taro (Figure 10 a). The controls remained asymptomatic. When the sources of variation were analyzed separately, there were significant differences between plants (Figure 11a). The size of the lesion in Peruvian carrot was higher than taro plants. Moreover, mortality was observed in Peruvian carrot plants on treatments inoculated with isolated LP9 (Figure 10 b). The isolate LP9 was recovered from the infected tissues, completing the Koch's postulates.

For the pathogenicity test using roots and tubers, there was no interaction between host × isolates (P = 0.927). When the sources of variation were analyzed separately, lesion size was differed only by the host (Figure 11b). Lesion length on Peruvian carrot roots was greater than lesion length on taro roots, regardless the isolates used (Figure10 c,d,e,f).

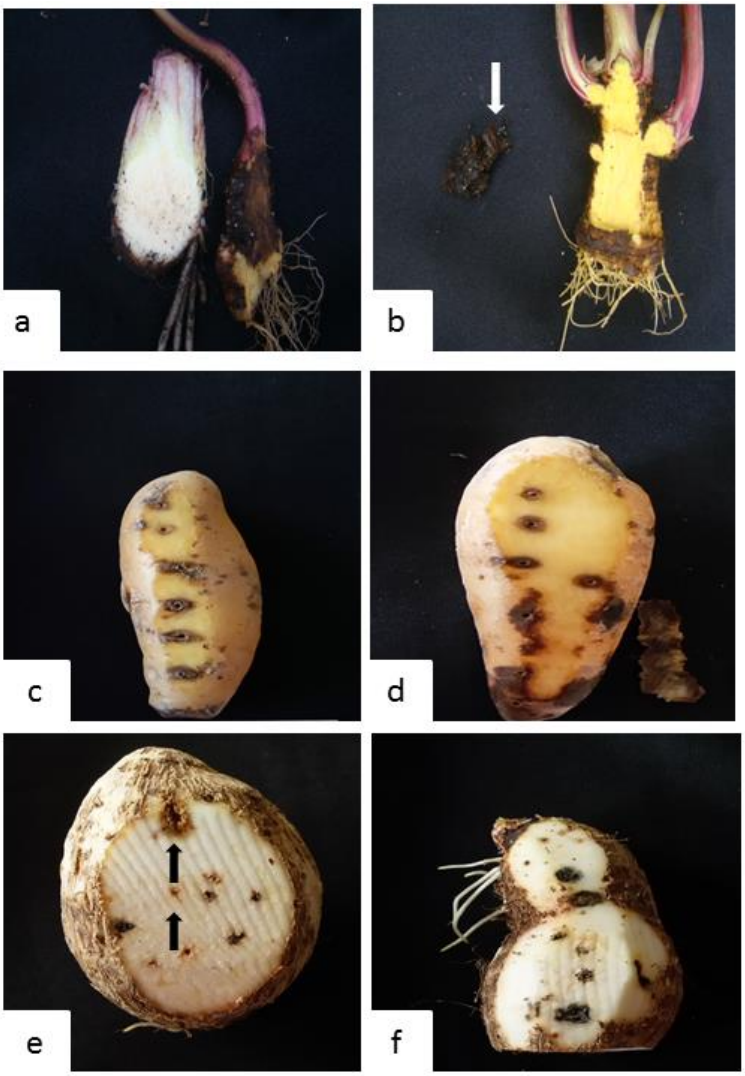

Figure 10 **a** Inoculated seedlings of taro (right) with isolated *Ceratocystis arracacicola* no symptoms and inoculated seedlings of Peruvian carrot with isolated *Ceratocystis arracacicola* with symptoms (left); **b**, Death of inoculated seedlings (right) and control uninoculated (left); **c**, *Arracacia xanthorriza* roots inoculeted with isolate the peruvian carrot (LP9); **d**, *Arracacia xanthorriza* roots inoculeted with isolate the taro (C1905); **e**, *Colocasia esculenta* tubers inoculeted with isolate the peruvian carrot (LP9) (Arrow indicates reddish halo); **f**, *Colocasia esculenta* tubers inoculated with isolate the taro (C1905).

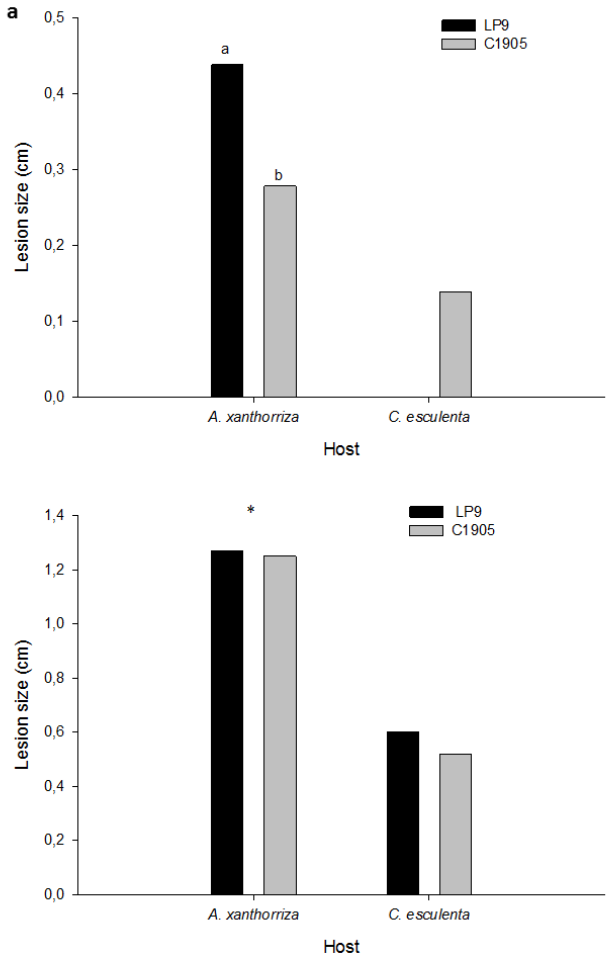

Figure 11 a, Lesion size after cross-inoculation with isolate the peruvian carrot (LP9) and one isolate the taro (C1905) in seedlings of *Arracacia xanthorriza* (A) and *Colocasia esculenta* (C); **b**, Lesion size after inoculation with isolate the peruvian carrot (LP9) and one isolate the taro (C1905) in *Arracacia xanthorriza* (A) roots and *Colocasia esculenta* (C) tubers. Means followed by different letters differ by Tukey test ($p<0.05$).

DISCUSSION

Isolates of *Ceratocystis* on Peruvian carrot were defined as new species receiving the name of *C. arracacicola*. The classification was based primarily on the results of phylogeny, morphology, interfertility and pathogenicity to the host. The sequence of the ITS region confirmed that isolates on Peruvian carrot belongs to the Latin America Clade (LAC). Of the four groups in which the genus *Ceratocystis* it was divided (Latin America, North America, Africa and Asia (Harrington 2000; Heath et al., 2009; Johnson et al., 2005; Thorpe et al., 2005)), the LAC is the largest of the groups which is *Ceratocystis fimbriata* attacking various hosts considered native or not. The rDNA ITS region has been useful in identifying the clade where isolates belong, but only this region has not have sufficient reliability to describe new species (Harrington et al.,2011; Harrington et al., 2014; Oliveira et al., 2015).

In the phylogenetic analysis generated for all genomic regions, only BT showed no clustering of isolates on Peruvian carrot in distinct clade. The ITS, MAT1 and MAT regions were supported by high bootstrap values and posterior probability, especially MAT1 and MAT2 regions, which when combined produce robust phylogenetic trees for the

C. fimbriata complex (Harrington et al. 2014). BT region in this study was not considered for classification of the species, since it grouped the Peruvian carrot isolates along with isolates of *C. platani* and one of the important features of *C. platani* is they have the expertise native host (*Platanus* spp) and was not able to come across with isolated of *C. fimbriata* of Brazil in other studies (Baker et al. 2003; Engelbrecht and Harrington 2005; Ferreira et al. 2010). Despite this information, Fourie et al. (2015), to evaluate molecular markers delimit the cryptic species in *Ceratocystis*, noted that BT 1 region were informative and significantly more useful than other regions. According to Harrington et al. (2011), Harrington et al. (2014) and Oliveira et al., 2015 is the ITS region that presented problems in the definition of the *Ceratocystis* species of LAC. Harrington et al. (2014) proved to be dubious taxonomic classification of new species *C. manginecans*, *C. mangicola*, *C. mangivora*, *C. eucalypticola* and *C. acaciivora* that were defined only by ITS sequences. Subsequently Oliveira et al. (2015) demonstrated by phylogenetic analyzes of some genes (including the mating (MAT1 and MAT2)), microsatellite analyses, and also by crosses tests, the new species described on mango (*C. manginecans, C. mangicola, C. mangivora, C.*

acaciivora) really were a unique biological and synonymous species *C. fimbriata*, as alerted by Harrington et al. (2014).

The results of the mating experiments were consistent with earlier studies (Engelbrecht et al. 2005; Ferreira et al. 2010; Harrington and McNew 1997, 1998; Johnson et al. 2005; Oliveira et al., 2015), and were considered indicative of an interspecific hybrid cross (H), supporting strongly *C. arracacicola* as a new species of the Latin American clade of *C. fimbriata*.

Cylindrical conidia of peruvian carrot isolate presented a little morphological difference compared to others isolates from Brazil. The differences were observed in the wall of conidia tips, characterized by a thicker wall. Engelbretch & Harrington (2005) also showed slight but significant morphological differences, related presence or absence of a doliform (barrel-shaped) conidial state and minor differences in size of perithecial bases and necks and ascospores, between three *Ceratocystis* isolates associated with cacao (*Theobroma cacao*), sweet potato (*Ipomoea batatas*), and sycamore (*Platanus* spp.). The few differences in morphology suggesting the evolution and divergence of these species has been primarily driven by host specialization. Cross-inoculation of

Peruvian carrot isolate did not affected taro plants, but high mortality was observed on Peruvian carrot using the isolate of the respective host. In the roots and tubers, we observed defense response in taro when inoculated with Peruvian carrot, which was characterized by reddish halo around the lesion. Baker et al. (2003) showed that *Ceratocystis* isolates on cacao, sweet potato and sycamore are highly specialized to their respective hosts in inoculation studies.

Although *C. arracacicola* have shown specialization to the host and is still restricted to a small area, the spread of isolates to another area and its adaptability to new areas free of the disease can cause selection pressure on other population and become problem not only for Peruvian carrot, but also for other species economically important.

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(VERSÃO PRELIMINAR)