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Diaporthe endophytica and *D. terebinthifolii* from medicinal plants for biological control of *Phyllosticta citricarpa*

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ABSTRACT

The citrus industry is severely affected by citrus black spot (CBS), a disease caused by the pathogen *Phyllosticta citricarpa*. This disease causes loss of production, decrease in the market price of the fruit, and reduction in its export to the European Union. Currently, CBS disease is being treated in orchards with various pesticides and fungicides every year. One alternative to CBS disease control without harming the environment is the use of microorganisms for biological control. *Diaporthe endophytica* and *D. terebinthifolii*, isolated from the medicinal plants *Maytenus ilicifolia* and *Schinus terebinthifolius* have an inhibitory effect against *P. citricarpa in vitro* and in detached fruits. Moreover, *D. endophytica* and *D. terebinthifolii* were transformed by *Agrobacterium tumefaciens* for *in vivo* studies. The transformants retained the ability to control of phytopathogenic fungus *P. citricarpa* after transformation process. Furthermore, *D. endophytica* and *D. terebinthifolii* were able to infect and colonize citrus plants, which is confirmed by reisolation of transformants from inoculated and uninoculated leaves. Light microscopic analysis showed fungus mycelium colonizing intercellular region and oil glands of citrus, suggesting that these two new species are capable of colonizing citrus plants, in addition to controlling the pathogen *P. citricarpa*.

1. Introduction

The fungus *Phyllosticta citricarpa* McAlp (sexual state: *Guignardia citricarpa* Kiely) is the etiological agent of citrus black spot (CBS) disease. With the deletion of Art. 59 from the International Code of Nomenclature for algae, fungi, and plants (ICN), asexual and sexual names of fungi received equal status (Hawksworth et al., 2011; Wingfield et al., 2012). *Phyllosticta* is adopted in the present study for this group of fungi because the name *Phyllosticta* (1818) predates *Guignardia* (1892) (Glienke et al., 2011).

The CBS disease has caused innumerous losses in citrus-producing regions worldwide. It is characterized by lesions on the fruit peel of sweet oranges, lemons, tangerines, and their hybrids (Marques et al., 2012; Aguiar et al., 2012). This disease depreciates the commercial value of the fruit, reduces crop productivity, and increases the production costs considerably, causing heavy monetary loss because of reduction in export of fresh fruits from Brazil and several other countries (Wulandari et al., 2009).

CBS disease control relies on the use of protective fungicides (Schutte et al., 1997). The pesticide concentration has been increased because of the emergence of mutant resistant pathogens, raising the cost of production. Furthermore, the use of these pesticides is a threat to the environment and human health. The use of pesticides is a major international concern that has led to the development of alternative techniques for agricultural sustainability (Niu et al., 2014). One economic, low impact option is biological control. Biological control using endophytes is promising, since they provide benefits to the host and colonize the same niche as pathogens (Miller et al., 2012; O'hanlon et al., 2012).

There are two different approaches for using endophytic microorganisms for alternative control of phytopathogens. The first one is to use secondary compounds produced by microorganisms for disease control and the second is to directly use the microorganism to control the pathogen (O'hanlon et al., 2012).

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Study of plant-microorganism interactions and pathogen-biological agent interactions by using reporter genes expressing fluorescent proteins, such as *DsRed* and *gfp* (Eckert et al., 2005), can help understand biological control by direct contact with microorganisms. One strategy for gene insertion in the fungal genome is genetic transformation by *Agrobacterium tumefaciens* (De Groot et al., 1998; Figueiredo et al., 2010). We successfully used this approach for inserting the gene *gfp* in *P. citricarpa* (Figueiredo et al., 2010). Furthermore, endophytic microorganisms of medicinal plants are currently being studied for potentially useful primary and secondary metabolites (Strobel, 2011).

Diaporthe species are the most frequently found endophytes in several plant species (Murali et al., 2006; Botella and Diez, 2011). This genus has also been recognized as a producer of several new compounds, and is of great interest in biotechnology (Elsaesser et al., 2005; Zang et al., 2012). Gomes et al. (2013) described two new species, *Diaporthe terebinthifolii* and *D. endophytica*, as endophytes of the medicinal plants *Schinus terebinthifolius* and *Maytenus ilicifolia*, respectively. The study aims to evaluate the potential of *D. terebinthifolii* and *D. endophytica* for biological control of the phytopathogen *P. citricarpa* and in evaluating CBS disease symptom control in detached fruits. Furthermore, this study evaluates the ability of these species to colonize endophytically in citrus plants by using the *dsred* reporter gene.

2. Materials and methods

2.1. In vitro tests with endophytic isolates

The antagonistic activity of the endophytic isolates *D. endophytica* (LGMF928 and 935) and *D. terebinthifolii* (LGMF907 and LGMF914) against the fungus *P. citricarpa* (LGMF05 and LGMF06) was evaluated using the paired culture technique described by Köhl et al. (1997). We used a randomized design with five replications. Each sample was presented in a Petri dish. Negative control received only *P. citricarpa*. To determine inhibition percentage (IP) the diameter of the colonies was measured, and the IP was calculated according to the formula: IP = mycelial growth in control − mycelial growth in treatment/mycelial growth in control × 100 (Quiroga et al., 2001). The active microorganisms were selected to verify if the inhibition was caused by the production of non-volatile or volatile metabolites, according to Morris et al. (2010). The endophytes that showed production of non-volatile metabolites were selected for the production of extracts for further tests.

2.1.1. Production of extracts

Metabolic extracts of the *Diaporthe* endophytes were obtained by fermenting five mycelia discs (Ø 8 mm) in 500 mL of malt extract medium (20 g of malt extract, 20 g of glucose, 1 g of peptone, and 1000 mL distillated water), under agitation for 28 days (120 rpm, 28 °C) according to Rodrigues et al. (2000). After fermentation, the mycelium was separated from the fermented liquid by Whatman n°4 filtration paper. The extraction was performed using ethyl acetate (EtOAc) (Merck). Solvent evaporation was carried out using a rotaevaporator at 45 °C. The final extract was weighed and diluted in methanol at a concentration of 10 mg/mL.

2.1.2. Activity of the extracts against the phytopathogen P. citricarpa

Antagonistic activity of the *D. endophytica* (LGMF928 and LGMF935) and *D. terebinthifolii* (LGMF907 and 914) extracts against *P. citricarpa* was evaluated by two approaches (*in vitro* and *in vivo*). The first methodology (*in vitro*) evaluated the pycnidial production of *P. citricarpa* in the surface of autoclaved citrus leaves, in the presence and absence of *Diaporthe* extracts. For this evaluation, we used Petri dishes with water agar medium and leaf discs (Ø 10 mm) containing 10 μL of the extract to be evaluated. Four discs of 2-mm-thick *P. citricarpa* mycelia were inoculated close to each leaf fragment. The Petri dishes were sealed and maintained at 28 °C, with 12 h photoperiod for 21 days. After this period, the pycnidia of *P. citricarpa* formed above the leaves were counted under a stereoscopic microscope. We used a randomized design with three replications.

The second methodology (*in vivo*) evaluated the potential of hydrophobic extract in inhibiting the development of lesion in detached orange fruits (Goulin et al., 2011). In this test, we used *Citrus sinensis* fruit and the *P. citricarpa* isolate LGMF06. The mycelium of LGMF06 was introduced in the fruit using a wound with cutting drill. To evaluate the extract activity against the pathogen, 10 μL (10 mg/mL) of extract was added to the wounds. The wound was sealed with tape and maintained in light chamber at 28 °C in continuous light. The qualitative activity was evaluated after 21 days of incubation, comparing the development of lesions in the treatments with and without the extracts application to the negative control (methanol).

2.2. Agrobacterium tumefaciens-mediated transformation

2.2.1. Strains, plasmid, and growth conditions

Agrobacterium tumefaciens strain EHA105 (Hood et al., 1993) harboring the binary vector pCAMDsRed with the *DsRed*-Express gene, hygromycin gene conferring resistance to hygromycin *(hph),* and the neomycin phosphotransferase II gene conferring resistance to kanamycin were considered for selection of bacteria [11]. *Agrobacterium tumefaciens* was grown at 28 °C in yeast extract peptone (YEP) supplemented with 100 mg/mL kanamycin. *D. endophytica* (LGMF-928, LGMF-935) and *D. terebinthifolii* (LGMF-907, LGMF-914) strains were stored in PDA, pH 5.8, and are currently part of the Culture Collection of the Laboratory of Microorganisms (LabGeM) at the University Federal of Paraná, Curitiba, Paraná, Brazil (http://www.labgem.ufpr.br/portal).

2.2.2. Agrotransformation

D. endophytica and *D. terebinthifolii* were transformed by *A. tumefaciens* according to the protocol described elsewhere (Sebastianes et al., 2012).

2.2.3. Transgene stability in D. endophytica and D. terebinthifolii

To confirm the genetic stability of the transformants, 11 transformants of *D. terebinthifolii* and 15 of *D. endophytica* were cultured on PDA without hygromycin B for five successive subcultures and then transferred to PDA containing hygromycin B (100 μg/mL). Transformants were considered stable when they remained resistant to hygromycin B after five subcultures (Fitzferald et al., 2003).

2.2.4. Expression analysis of the reporter DsRed

Twelve transformants were randomly selected and cultured on PDA with hygromycin B (100 μg/mL) and were incubated at 28 °C for 1–4 days. The red fluorescence emission associated with DsRed was detected using a fluorescence microscopy Leica UV (DMKLB or MZFL111) with the following filter settings: 488 nm excitation and 515 nm emission (Eckert et al., 2005). Images were recorded and processed using Picasa 3.1.0 software. The wild type strains were used as negative controls.

2.2.5. PCR assays

For the transgenes detection in *D. endophytica* and *D. terebinthifolii* by PCR, total DNA was extracted from mycelium grown on PDA medium for 3 days at 28 °C. The mycelium was harvested and grounded with mortar and pestle under liquid nitrogen. Genomic DNA was obtained according to

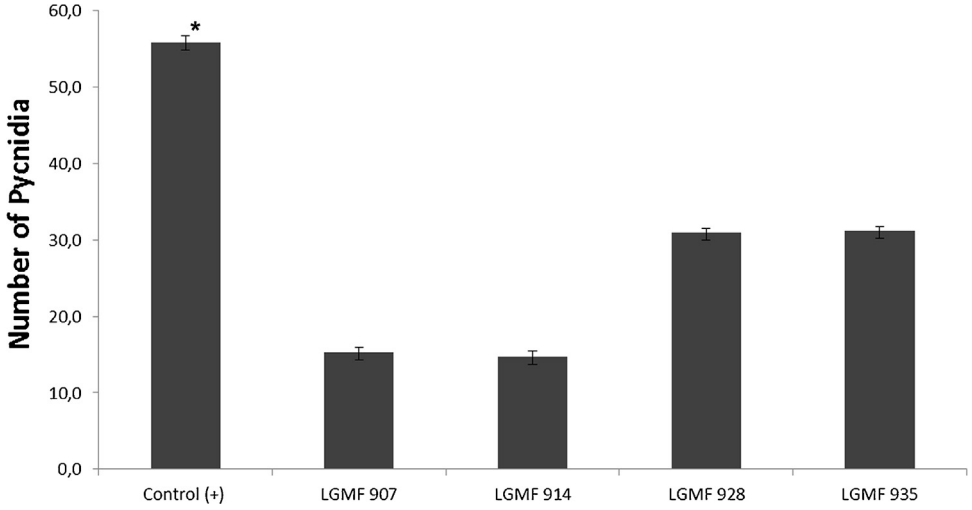

Fig. 1. Activity of the extracts against the phytopathogen *P. citricarpa in vitro*. *P. citricarpa*, LGMF05 (Control +) pycnidium production in citrus leaves after 28 days of incubation using 10 μL of the methanolic extracts of *D. endophytica* (LGMF928 and LGMF935) and *D. terebinthifolii* (LGMF907 and LGMF914). Average of pycnidium number. Methanolic extract concentration (10 mg/mL). ($p < 0.01$ Tukey's test).

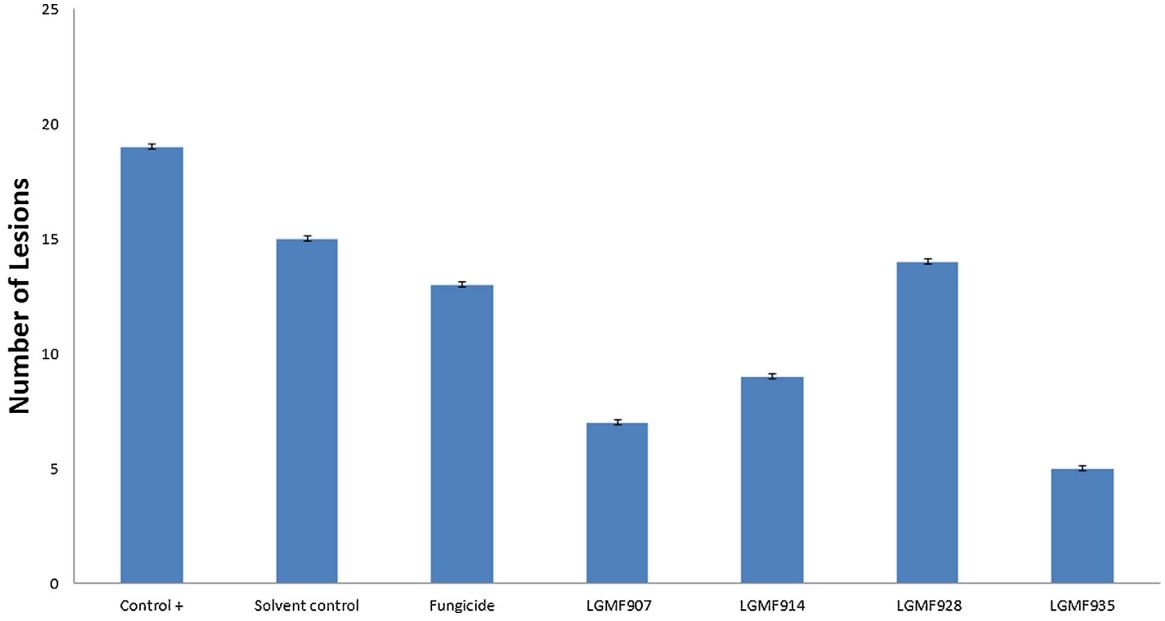

Fig. 2. Activity of the extracts against the phytopathogen *P. citricarpa in vivo*. Symptoms of citrus black spot lesions on detached fruits (*Citrus sinensis*Osbeck). Development of lesions when inoculated with *P. citricarpa* (Control +); *P. citricarpa* + methanol (Solvent control); *P. citricarpa* + 10 μl Derosal® (1.0 mg/mL) (Fungicide); *P. citricarpa* + 10 μL methanolic extract (100 μg/mL): LGMF907 (Methanolic extract LGMF907), LGMF914 (Methanolic extract LGMF914), LGMF928 (Methanolic extract LGMF928), LGMF935 (Methanolic extract LGMF935). Nonparametric ($p < 0.05$ Kruskal-Wallis).

the methods described by Gomes et al. (2013). The amplification reactions of 25 selected transformants were performed using the primers hph1 (5′AGCGTCTCCGACCTGATG3′) and hph2 (5′CGACGGACGCACTGACGG3′) and conditions described by Malonek and Meinhardt (2001). PCR products were resolved by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light (Ultraviolet Benchtop transilluminators) and photographed (Digi doc it software). The wild-type strains (WS) were used as the negative control.

2.3. *Inoculation of* D. terebinthifolii *and* D. endophytica *in citrus plants*

Citrus plants (16) with two years of growth and a height of 25 cm were selected from a green house. The endophytes LGMF907-T2, LGMF914-T2 (*D. terebinthifolii*), LGMF928-T9, and LGMF935-T5 (*D. endophytica*) were inoculated after the leaves suffered an injury in the adaxial region. The isolates were grown on potato-dextrose agar (PDA) 28 °C with cellophane for 5 days. After this, 2 cm^2 mycelium was scraped and diluted in distilled water, and 20 μL of this solution was inoculated on leaves at the point of injury. Inoculation was performed after injury of the sheets, since previous experiments showed the absence of infection when fragments without injury were used.

The leaves were then evaluated by isolation of endophytic from leafs tissue and also by light microscopy.

2.4. *Isolation of endophytic transformant strains from citrus plants*

The evaluations were performed after 7, 14, 21, and 28 days, and monthly for one year after inoculation. The leaves were

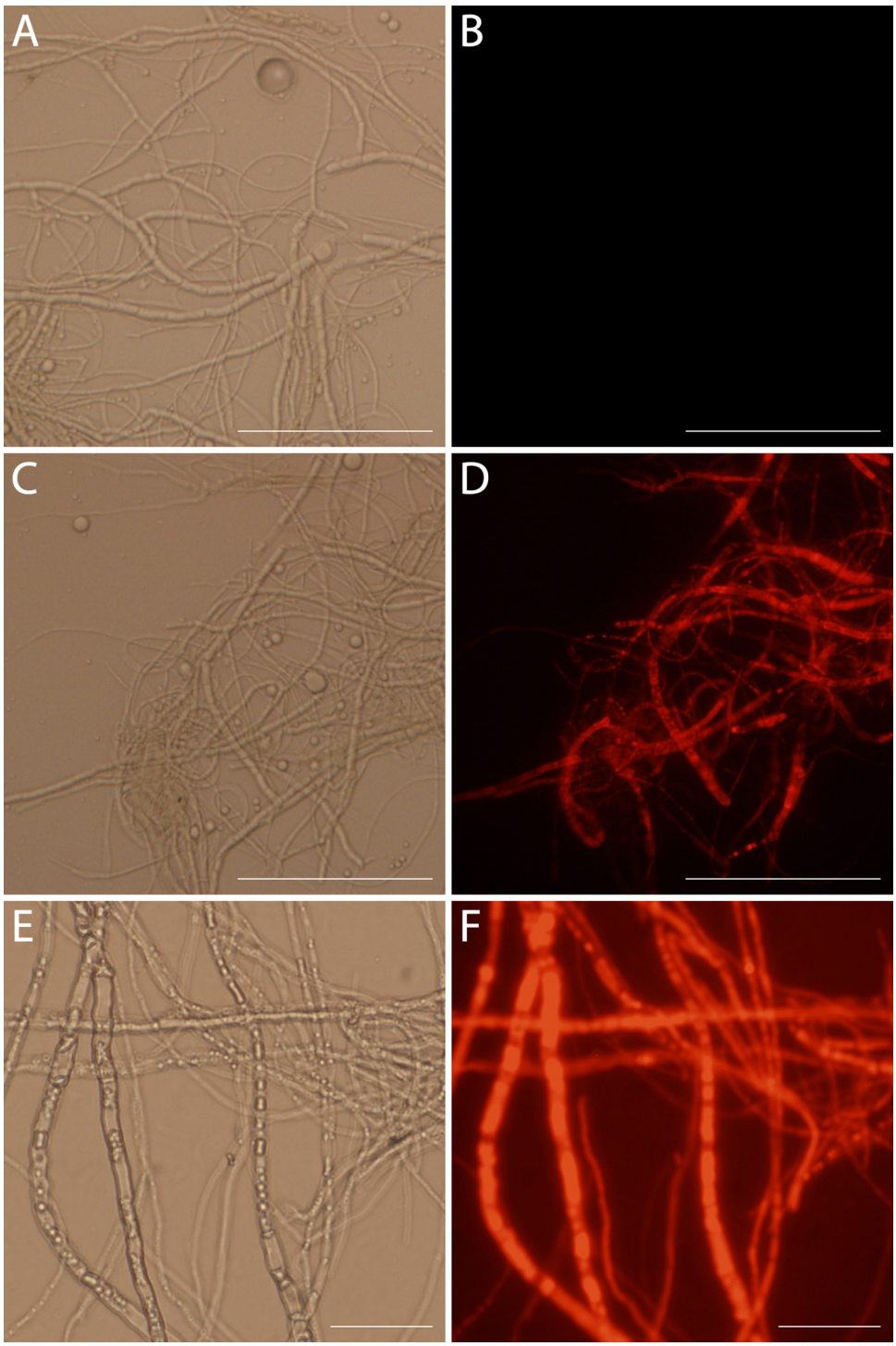

Fig. 3. Photomicrographs of wild and transformants strains of *Diaporthe terebinthifolii* and *Diaporthe endophytica.* Epifluorescence microscopy images of wild-type *D. terebinthifolii* LGMF907 (A–B); *D. terebinthifolii* LGMF907.T2 (C–D), *D. endophytica* LGMF928.T9 (E–F). Scale bar: 50 μm.

detached and washed in water, and then disinfection for epiphytic microorganisms was done as described by Petrini (1986). Small fragments (8 × 8 mm) were then plated on PDA medium containing 100 μg/mL of tetracycline. The plates were incubated at 25 °C for 30 days, and the growth was checked daily to ensure that the ringing of the microorganisms has developed. The endophytic isolates with similar morphology of inoculated fungi were subcultured on new plates for further fluorescence verification.

2.5. Morphophysiological analysis of citrus leaves

After the disinfection process according to Petrini (1986), for the anatomical studies, fixed samples were dehydrated in a graded ethanol series and embedded in methacrylate (Historesin, Leica Instruments, Germany). Transverse and longitudinal sections (5 μm thick) were obtained with an automated advance rotating microtome (RM2155, Leica Microsystems Inc., USA) and stained with toluidine blue at pH 3.2 for structural characterization. Anal-

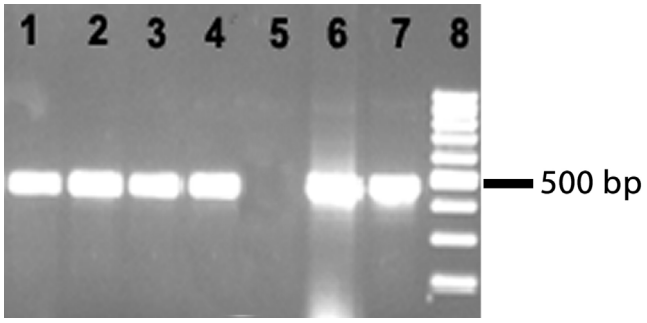

Fig. 4. Agarose gel electrophoresis showing presence of the gene *hph* in *D. terebinthifolii* LGMF907.T2, LGMF914.T2 (1–2), *D. endophytica* LGMF928.T9, and LGMF935.T5 (3, 4). Isolated wild type was used as a negative control (5), plasmid PCAMDsRed was used as a positive control (6–7), and 100-bp ladder (8).

ysis of the slides was performed under a light microscope (Zeiss Axioskop 2) by taking photographs with a digital camera.

2.6. Statistical analysis

Analyses of variance (ANOVAs) of the results were conducted. If the *F* test was significant, posteriori comparisons were carried out using the Tukey's test. To analyze the experiment results for fruits the one-way nonparametric Kruskal–Wallis was used. All experiments were analyzed separately.

3. Results

3.1. In vitro tests with endophytic isolates

We evaluated two isolates of *D. endophytica* (LGMF928 and LGMF935) and two isolates of *D. terebinthifolii* (LGMF907 and LGMF914) *in vitro* against the phytopathogen *P. citricarpa*. Paired cultures revealed that these four isolates inhibited more than 70% of the *P. citricarpa* (LGMF05 and LGMF06) mycelial growth after 14 days of incubation (Table 1). Using *in vitro* tests, we found that the inhibition of *P. citricarpa* mycelial growth by endophytic *Diaporthe* spp. strains observed in paired cultures was caused mostly by non-volatile metabolite production (Table 1).

Table 1
The antagonistic activity of endophytic isolates *D. endophytica* (LGMF928 and 935) and *D. terebinthifolii* (LGMF907 and LGMF914) against the fungus *P. citricarpa* (LGMF05 and LGMF06).

Tratament	Growth rate (cm) after 14 days		
	Dual culture	Non-Volatile Metabolites	Volatile Metabolites
LGMF05	3.5	4.1	3.3
LGMF06	3.01	2.02	1.9
LGMF05 + LGMF928	0.8	1.0	2.6
LGMF06 + LGMF928	0.86	0.97	1.4
LGMF05 + LGMF935	0.9	1.3	2.9
LGMF06 + LGMF935	0.87	1.42	1.1
LGMF05 + LGMF907	1.0	0.9	2.6
LGMF06 + LGMF907	0.72	0.62	1.59
LGMF05 + LGMF914	0.8	0.6	2.2
LGMF06 + LGMF914	1.25	0.7	1.17

3.2. Activity of the extracts against the phytopathogen P. citricarpa *in vitro*

Extracts from endophytic strains (LGMF907, LGMF914, LGMF928, and LGMF935) caused reduction in the number of pycnidia produced by *P. citricarpa* (LGMF05) on the surface of the autoclaved citrus leaves (Fig. 1). *D. terebinthifolii* showed the highest inhibition of more than 70%.

3.3. Activity of the extracts against the phytopathogen P. citricarpa *in vivo*

The inhibitory action of the extracts was also evaluated *in vivo* by the CBS symptom induction methodology in detached fruits. The extracts from *D. endophytica* and *D. terebinthifolii* inhibited

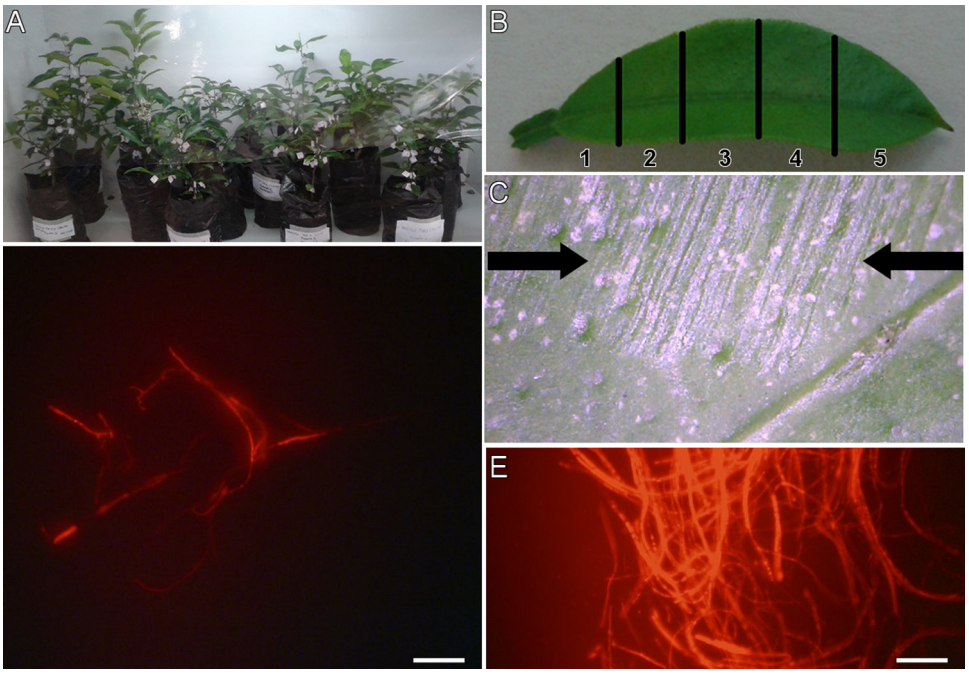

Fig. 5. Inoculation and reisolation of *D. terebinthifolii* and *D. endophytica* from citrus plants. Citrus seedlings inoculated with the DsRed transformant *Diaporthe* spp. (A); Citrus leaf inoculated in fragment "3" (B); Leaf injured by needle tip, between arrow. Region of inoculation (C); Epifluorescence microscopy image of *D. terebinthifolii* (LGMF907.T2) isolated from citrus seedling (D); Epifluorescence microscopy image of *D. endophytica* (LGMF928.T9) isolated from citrus seedling (E). Scale bar: 50 μm.

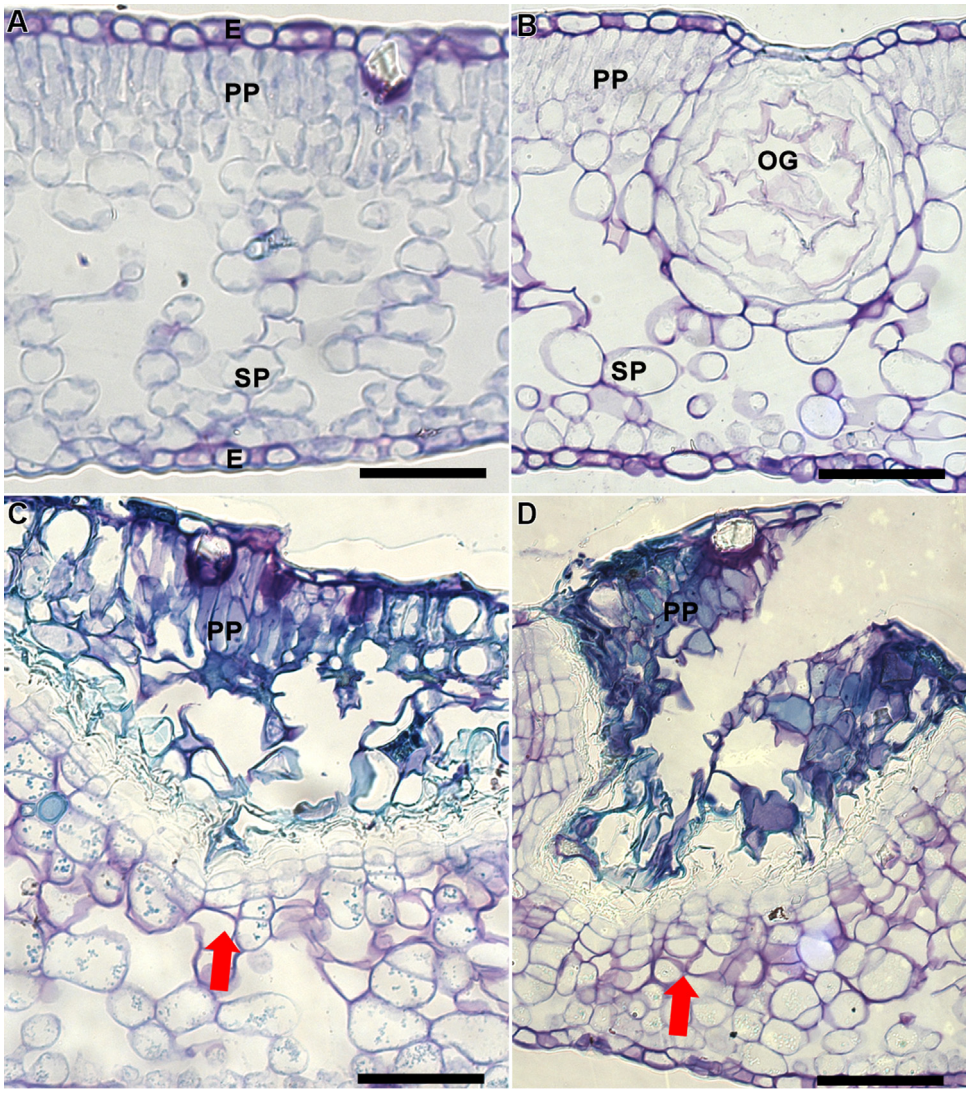

Fig. 6. Cross-sections of healthy citrus leaves and damaged citrus leaves. Mesophyll cells presenting intact tissues and numerous intercellular spaces (A, B); healing following injury in orange fruit by "nail file" (C); healing following injury in orange fruit by "sewing needle" (D); cells healing (arrow), palisade parenchyma (PP), oil gland (OG), epidermis (E), spongy parenchyma (SP). Scale bar: 50 μm.

the development of CBS lesions, wherein the number of lesions formed is lower using extracts from endophytic strains LGMF907 and LAGMF914 than observed in treatment using the fungicide Derosal® (Fig. 2).

3.4. *Agrobacterium tumefaciens*-mediated transformation

Out of the 26 transformants that showed genetic stability, 15 were of *D. endophytica* and 11 were of *D. terebinthifolii*. These transformants were morphologically similar to the wild strains, and were able to grow in selective medium (add hygromycin) and their hyphae showed red fluorescence (Fig. 3).

The transformants were evaluated by PCR using the primers hph1 and hph2 to confirm the insertion, and an amplicon of 500 bp representing the *hph* gene was obtained (Fig. 4). We selected four transformants (LGMF907.T2, LGMF914.T2, LGMF928.T9, and LGMF935.T5) for the subsequent experiments. Initially, these transformants were evaluated for their ability to inhibit *P. citricarpa* and to assess whether the transformation silenced any gene involved in the biological control activity. The four isolates tested inhibited the fungus *P. citricarpa* and there was no difference in their activities.

3.5. *Colonization of* D. terebinthifolii *and* D. endophytica *in citrus plants*

The ability of *D. terebinthifolii* and *D. endophytica* to colonize citrus plants was evaluated. The endophyte was inoculated in 16 citrus plants and the evaluation was performed by reisolation experiments at different times after inoculation, followed by fluorescence microscopy of the isolates. A total of 128 leaves from 16 plants were evaluated, and *Diaporthe* spp-DsRed was isolated from all of the analyzed plants (Fig. 5). Out of the 38 leaves expressing DsRed, nine and ten leaves were colonized by LGMF907.T2 and LGMF914.T2, respectively (*D. terebinthifolii*), and eleven and eight were colonized by LGMF928.T9 and LGMF935.T5 (*D. endophytica*), respectively. The transformants were reisolated from injured leaves. No strain was obtained from leaves of non-inoculated plants that were maintained close to inoculated plants (negative control), indicating that the fungus has no airborne dispersion.

3.6. *Morphophysiological analysis of citrus leaves*

Citrus leaves are hypostomatic. The epidermis cells are flat, juxtaposed with prominent vacuole, with a thicker cuticle on the

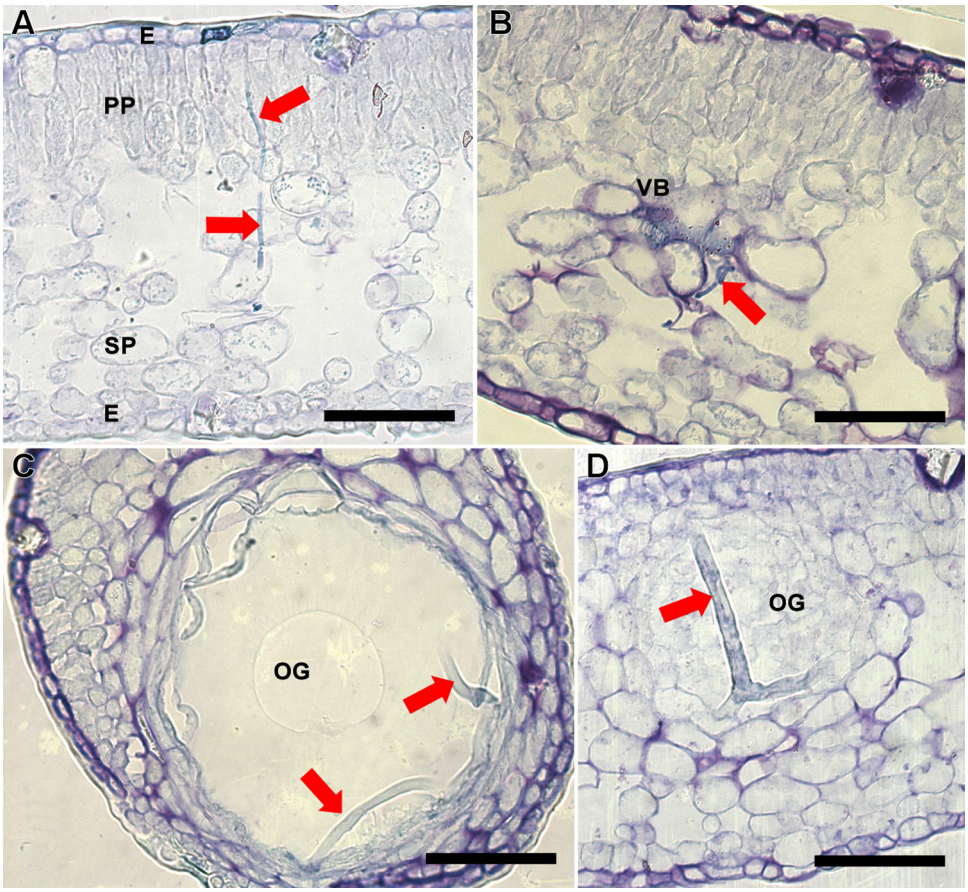

Fig. 7. Cross-sections of citrus leaves demonstrating the colonization by species of *Diaporthe*. The arrow indicates the mycelium of *D. endophytica* (LGMF935.T5) colonizing mesophyll cells, in the area of intercellular palisade parenchyma and spongy parenchyma (A); mesophyll cells with mycelium (LGMF935.T5) colonization in the intercellular area near the vascular bundle (arrow) (B); arrow indicates the mycelium of *D. terebinthifolii* (LGMF907.T2) colonizing the oil gland (C); arrow indicates mycelium (LGMF935.T5) colonization in the oil gland in the intercellular area of the rib (D). Palisade parenchyma (PP), oil gland (OB), epidermis (E), vascular bundle (VB), spongy parenchyma (SP). Scale bar: 50 μm.

adaxial side (Fig. 6A and B). Scar tissue formed in the region of inoculation because of injury. Cells formed in this region covered the gap formed by the injury and developed parenchyma cells with intercellular spaces (Fig. 6, C and D). These spaces probably aided in the penetration and hyphal growth of the inoculated fungus.

Analysis of images from the inoculated tissue region and nearby regions showed hyphae of both species (*D. terebinthifolii* and *D. endophytica*) colonizing intercellular spaces (Fig. 7). This result suggests that the fungus do not colonize leaf cells, as expected for endophytic colonization.

4. Discussion

Every plant species examined to date harbors endophytic fungi within its asymptomatic aerial tissues, such that endophytes represent a ubiquitous, yet cryptic, component of terrestrial plant communities (Ardanov et al., 2012). The endophytic isolates used in this study were isolated from Brazilian medicinal plants, *S. terebinthifolius* and *M. ilicifolia*, commonly used to treat bacterial and viral infections (Gundidza et al., 2009). Endophytic microorganisms can reduce herbivory or phytopathogen settling, promoting plant growth and inducing systemic resistance in plants (Miller et al., 2012; O'hanlon et al., 2012). Thus, endophytic protection can provide an alternative to traditional pesticide treatment (Niu et al., 2014; Coloff et al., 2013) and can be used as for biological control (Ardanov et al., 2012). Hence, we evaluated the capacity of endophytic strains to control CBS disease.

The metabolites produced by *D. terebinthifolii* and *D. endophytica* showed high inhibition to *P. citricarpa* pycnidium formation (Fig. 1), especially from the strains LGMF907 and LGMF914 (inhibition over 70%). These results are important with respect to disruption of the disease cycle of CBS, wherein the asexual stage of fungal growth is responsible for aggravating the disease within the plant and surrounding areas (Perryman et al., 2014). Strategies to minimize the appearance of *P. citricarpa* pycnidia in citrus are of fundamental importance and represent a breakthrough in disease control research. *In vivo* analysis showed that the metabolites produced by *D. terebinthifolii* (LGMF907 and LGMF914) and *D. endophytica* (LGMF935) are more effective in inhibiting CBS symptoms than the fungicide Derosal®, commonly used in citrus crops. This study has significant importance, as there is no effective treatment for CBS disease and the problems related with the presence of fungicides residues in fruits (Schreiber and Bailey, 2012).

Furthermore, we evaluated the ability of these endophytes expressing the reporter gene for red fluorescence (DsRed) to colonize citrus plants. Such transformants were successfully obtained using *Agrobacterium tumefaciens*. This protocol was effective and simple and directly used the mycelium, which is important for fungi with no reproductive structures. The transformants showed no morphology and/or physiology differences from the wild strains (LGMF907.T2; LGMF914.T2; LGMF928.T9; LGMF935.T5) (data not shown). Genetic transformation of filamentous fungi is an important tool to study their interaction with several plants, and their interaction with other microorganisms occupying the same niche

(Schreiber and Bailey, 2012; Li et al., 2013). Before the colonization test, it was ensured that such transformants preserve the inhibitory capacity compared to the wild strain. *Diaporthe* spp. transformants inhibited the *P. citricarpa* mycelial growth higher than 95%, 21 days after inoculation.

Our results showed that DsRed transformants of *D. endophytica* and *D. terebinthifolii* were able to endophytically colonize citrus seedlings. This was verified by re-isolation of strains expressing *DsRed* from all inoculated plants evaluated, including leaves that had not been inoculated. Furthermore, it was found that strains remained viable in plants even 12 months after inoculation. Anatomic evaluations of the cross section of leaf tissues isolated from the inoculated region showed the presence of fungal mycelium colonizing the intercellular region and in the oil glands. These data confirm the results obtained in reisolation experiments. Reporter genes have been widely used in studies determining the ability of fungi to establish endophytic interactions with the host plant (Favaro et al., 2012) and can be used to study the interaction of antagonistic strains (Mikkelsen et al., 2003).

Here, we demonstrate the capacity of *D. endophytica* and *D. terebinthifolii* strains to produce secondary compounds acting directly against *P. citricarpa*, pycnidium formation, and CBS symptoms. Moreover, we suggest that these endophytes are able to efficiently colonize citrus plants and that they can remain viable within the plant for a long time without causing disease symptoms. The ability of these endophytes to colonize citrus leaves and their production of metabolites that inhibit the pycnidium formation and CBS lesions suggest that these strains can provide an important strategy to control CBS disease. However, further study is necessary to evaluate the activity of endophytic isolates against *P. citricarpa in vivo*.

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