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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