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Agrobacterium-mediated transformation of Guignardia citricarpa: An efficient tool to gene transfer and random mutagenesis

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ABSTRACT

Guignardia citricarpa is the causal agent of Citrus Black Spot (CBS), an important disease in Citriculture. Due to the expressive value of this activity worldwide, especially in Brazil, understanding more about the functioning of this fungus is of utmost relevance, making possible the elucidation of its infection mechanisms, and providing tools to control CBS. This work describes for the first time an efficient and successful methodology for genetic transformation of G. citricarpa mycelia, which generated transformants expressing the gene encoding for the gfp (green fluorescent protein) and also their interaction with citrus plant. Mycelia of G. citricarpa were transformed via Agrobacterium tumefaciens, which carried the plasmid pFAT-gfp, contains the genes for hygromycin resistance (hph) as well as gfp. The optimization of the agrotransformation protocol was performed testing different conditions (type of membrane; inductor agent concentration [acetosyringone — AS] and cocultivation time). Results demonstrated that the best condition occurred with the utilization of cellulose's ester membrane; 200 μM of AS and 96 h as cocultivation time. High mitotic stability (82 %) was displayed by transformants using Polymerase Chain Reaction (PCR) technique to confirm the hph gene insertion. In addition, the presence of gfp was observed inside mycelia by epifluorescence optical microscopy. This technique easy visualization of the behaviour of the pathogen interacting with the plant for the first time, allowing future

studies on the pathogenesis of this fungus. The establishment of a transformation method for *G. citricarpa* opens a range of possibilities and facilitates the study of insertional mutagenesis and genetic knockouts, in order to identify the most important genes involved in the pathogenesis mechanisms and plant—pathogen interaction.

Introduction

Guignardia citricarpa Kiely (anamorph: *Phyllosticta citricarpa* McAlpine), an Ascomycete fungus, is the causal agent of Citrus Black Spot (CBS) disease in citrus plants. The most important symptoms caused by this disease are lesions on the rind of citrus fruits, which cause no internal decay (Kotzé 1981; Snowdon 1990; Smith *et al*. 1997), but fruits are depreciated in the fresh fruit market. Also, during fruit development, heavy infection close to the pedicel may induce premature fruit drop (Kotzé 1981; Smith *et al*. 1997) resulting in serious productivity losses.

Besides Brazil, the disease is present in countries like Argentina, Peru, Uruguay, South Africa, Mozambique, Kenya, Zambia, Zimbabwe, Japan, China, Hong Kong, Indonesia, Philippines, Taiwan, Australia, New Zealand (EPPO 2006), and recently, was reported at the United States of America (Adaskaveg *et al*. 2010; Schubert *et al*. 2010). This report was also investigated and confirmed by the Department of Agriculture of the United States (Adaskaveg *et al*. 2010). This disease has not been reported in North America thus far, marking a major spread of this pathogen in geographical context (Smith 2006).

At the European Union (EU), CBS is classified as A1 quarantine disease, which means that is not present in its member countries. Aiming to prevent the introduction of pathogens, they have a strong regulation that restricts the importation of citrus (Bonants *et al*. 2003). Detection of CBS disease in a single fruit prevents the landing of the good ones.

At CBS epidemic phase, *G. citricarpa* produces sexual and asexual spores. Pseudothecia along with ascospores are produced exclusively in decomposing leaves on the orchard floor (McOnie 1965; Kotzé 1981). Ascospores are released during rainfall events and are dispersed by wind. On the leaf surfaces in the presence of free water, ascospores infect the host by direct penetration and form a mycelial mass in the subcuticular region. The fungus then remains quiescent until the leaves fall or the fruit begins to ripen (Kotzé 2000).

Pycnidia with conidia are produced in hard spot and freckle spot fruit lesions as well as on dead branches and leaf litter prior to pseudothecia formation. When the pycnidia are mature, the conidia emerge from their ostiole covered by a mucilaginous substance and, in contact with water conidia are dispersed by splashing or being washed off by rain to nearby susceptible tissues, where new infections may occur (Kotzé 2000).

The underlying molecular mechanisms responsible for the complex symptomology have not yet been studied for *G. citricarpa*. Identification of plant infection and pathogenesis genes will enable us to address the key virulence aspects for this fungus and provide a foundation for better strategies to manage CBS. Analysis of gene functions can be accomplished *via* random mutagenesis or reverse genetic approaches. In this way, fungal transformation with heterologous DNA may result in random integration of the new genes into the fungal genome causing gene disruption as an insertional mutagenesis. The most important advantage of insertional mutagenesis over chemical or radiation mutagenesis is that the disrupted genes are tagged by the transforming DNA (T-DNA), which can be used to identify the disrupted genes (Sugui *et al*. 2005). Although classical genetic studies, such as parasexual and sexual recombination, had not been developed for *G. citricarpa*, functional genes related to plant infection, colonization, and pathogenesis can be identified through gene disruption by *Agrobacterium tumefaciens*-mediated transformation (ATMT), allowing the identification of genes related to these functions.

Many pathogenic fungi species and oomycetes have been already genetically transformated by this technique (Michielse *et al*. 2005; Lacroix *et al*. 2006). These results allow the study of the pathogenic behaviour of these fungi, such as the interaction with the plant, facilitating the comprehension of the pathogenic system, and the searching for control methods of disease. The plant—fungus interaction can also be studied by different approaches by microscopy techniques using reporter genes such as gene for green fluorescent protein (*gfp*) of the jellyfish *Aequorea victoria* (Lorang *et al*. 2001). The ATMT allow this kind of insertion as tool of study, and has been widely used for gene transfer and as a tool for insertional mutagenesis (Abdudeh *et al*. 2000; Blaise *et al*. 2007).

Michielse *et al*. (2005) describe different factors as determinants of processing efficiency, among them, the strain of *Agrobacterium*, concentration of acetosyringone (AS), conditions for cocultivation, and substrate composition.

Here we report the transformation of *G. citricarpa* mediated by *A. tumefaciens* for insertional mutagenesis, gene disruption. Also, we used a gfp tagged strain to study fruit colonization, showing that this is an important strategy to understand the pathogenesis of this fungus in citrus plants.

In this work, our major aims were:

1. To develop and optimise protocol for transformation of *G. citricarpa*
2. Evaluate transformation efficiency at different conditions of:
   a. Filter type
   b. Concentration of AS — bacterial virulence inductor agent
   c. Cocultivation period
3. To study the ability of *G. citricarpa* transformants to colonize the tissues of citrus fruit by microscopy technique
4. To check transformants for their infectiveness
5. To analyse enzymatic production modifications in transformants

Materials and methods

Strains, plasmids, and culture conditions

Guignardia citricarpa strain 12P strain was previously isolated from *Citrus sinensis* 'Valência' (Rodrigues *et al.* 2007). This strain and transformants obtained by ATMT were grown on potato-dextrose-agar (PDA, Oxoid) at 28 °C, for 15 d, and this collection is permanently conserved by Castellani method (Castellani 1963) at the 'Laboratório de Genética de Microrganismos Prof. Dr João Lúcio de Azevedo', University of São Paulo, Escola Superior de Agricultura 'Luiz de Queiroz', Department of Genetics, Piracicaba, São Paulo, Brazil. *Agrobacterium tumefaciens* strain EHA105 (kindly provided by Prof. Dr Carlos Alberto Labate − USP/ESALQ, Brazil) was grown in YEP (Chilton *et al.* 1974) supplemented with rifampicin (100 µg mL^{-1}) and spectinomycin (200 µg mL^{-1}). Transformants were selected on PDA medium supplemented with hygromycin (75 µg mL^{-1}), sodium cefoxitin (Claforan®) (300 µg mL^{-1}), and tetracyclin (125 µg mL^{-1}).

The transformation vector pFAT-gfp (Fitzerland *et al.* 2003) was kindly provided by Dr M. Kim Plummer (CSIRO Plant Industry, Australia). This vector contains in the T-DNA region, the hygromycin resistance gene from *Escherichia coli* (*hph*), under control of the *gpd* (glyceraldehyde-3-phosphate dehydrogenase) gene promoter of *Glomerella cingulata*, as well as the *gfp* gene (pGreen Lantern, Life Technologies), controlled by *gpd* promoter from *Aspergillus nidulans*.

ATMT transformation

The electrocompetent EHA105 strain of *Agrobacterium tumefaciens* was prepared according to Lacorte & Romano (1998) and transformed with pFAT-gfp vector. This transformed strain was grown on YEP broth in a rotatory shaker (180 rpm) at 28 °C for 24 h until obtaining an OD 600 nm of 0.8 and transformation was carried out in according to De Groot *et al.* (1998). Fungal cultures actively growing on filter paper on a PDA plate were transferred with the filter to IM medium (10 mM K$_2$HPO$_4$; 10 mM KH$_2$PO$_4$; 2.5 mM NaCl; 2 mM MgSO$_4$; 0.7 mM CaCl$_2$; 9 µM FeSO$_4$; 4 mM NH$_4$SO$_4$; 10 mM glicose; 0.5 % glicerol; 40 mM acid 2-[*N*-morfolin]-ethanosulfonic esterillized by filtration, pH 5,3), plus the bacterial inductor of virulence agent AS (Fluka), tested in concentrations of 200 and 400 µM; and covered with 2 mL of an induced *A. tumefaciens* EAH105 [pFAT-gfp] culture. The plates were incubated at 28 °C for 48 and 96 h for cocultivation. To select the ATMT transformants, filters containing the transformants were transferred to selection plates and grown for 60 d. Hygromycin-resistant colonies were subcultured onto PDA + HygB for five selective rounds. Different materials of filters to test the ability of the fungi to grown in it were observed. The filters used were cellulose's ester (Millipore®); cellulose (J. Prolab, Brazil); and nylon (Amersham Hybond N$^+$ or GE Healthcare).

DNA analysis

The total DNA from wild type strain and transformants was extracted by a standard phenol−chloroform protocol

according to Raeder & Broda (1985). To analyse the number of T-DNA copies integrated in *Guignardia citricarpa* genome, three randomly selected transformants were evaluated by Southern blot (Fig 2). Fifteen grams of genomic DNA from the wild type strain and the transformants, and 2 g of vector DNA pFAT-gfp, were digested with *Eco*RI restriction enzyme, according to the manufacturer's recommendations (Invitrogen, Brazil). These DNA were cleaved with restriction enzyme *Eco*RI, which cuts the T-DNA two times and do not cut inside the *gfp* gene, and transferred to nylon membranes and hybridized with a probe fragment of 700 bp of *gfp* gene. The T-DNA region of the pFAT-gfp vector has two recognition sites for this endonuclease, both located in the gene expression cassette for resistance to hygromycin B (Fitzerland *et al.* 2003). Thus, when the *hph* gene is used as a probe, all transformants as well as vector gfp-pFAT, will present a band of 1600 bp, and one or more bands of different sizes, depending on the number of T-DNA insertions. The digested DNA was separated on 1 % agarose gel and the standard procedures described by Sambrook & Russell (2001) were used to transfer the gel to nylon membrane (0.45 µm, Amersham Hybond N$^+$, GE Healthcare). The *hph* gene used as the probe was amplified from the vector pFAT-gfp with the primers hph1 and hph2, and purified with UltraClean™ Polymerase Chain Reaction (PCR) Clean-Up Kit (MOBIO Laboratories). The probe labelling and hybridization were performed using the thermostable alkaline phosphatase system (Amersham AlkPhos Direct™ Labeling and Detection Systems, GE Healthcare), under conditions of high stringency (60 °C), according to the manufacturer's recommendations.

Analysis of T-DNA flanking sequences

The regions flanking the T-DNA insertion were identified by TAIL-PCR technique (Thermal Asymmetric Interlaced Polymerase Chain Reaction) (Liu & Whittier 1995). For this, we used specific primers for left border (LB) and right border of the T-DNA vector pFAT-gfp (Fitzerland *et al.* 2003). The primers LB1-1 (5′-gtcagctccggcaccttatccttg-3′), LB2-2 (5′-cctcgttacatcagctcgcagctac-3′), LB3-1 (5′-cgtccgcaatgtgttataagttgtc-3′), and RB1 (5′-aagatgggcagtctttcagaaggg-3′), RB2-2 (5′-atcgcaaagtgaagtcttgctgcc-3′), and RB3-2 (5′-ccaaacgtaaaacggcttgtcccgc-3′) were used with arbitrary and degenerated (AD) primers. Specific primers for LB used in second and third reactions (LB2-2 and LB3-1, respectively) were placed at a distance of 105 bp from each other, to facilitate the confirmation of product specificity by comparing the size of bands generated.

Genomic DNA of transformants was extracted according to Raeder & Broda (1985) and used as template in the TAIL-PCR reactions. The AD primers (AD1, AD2, and AD3) used and the composition of the PCR reactions were those described by Liu & Whittier (1995). The TAIL-PCR amplification was performed following the conditions published by Mullins & Kang (2001). Amplification reactions were performed in a thermocycler PTC − 200 (MJ Research). For each transformant, the products generated in the first, second, and third reactions were analysed side by side on a 1.2 % agarose gel. All fragments obtained from the third reaction showed that decrease in length consistent with the position of the primers in the T-DNA (compared to

secondary reaction products) was considered as the junctions of T-DNA (Combier *et al.* 2003).

The products of the third reactions were purified from agarose gel, cloned into pGEM-T Easy vector (Promega, Brazil), and transformed into competent *Escherichia coli* DH5α cells. The amplification of the insert was performed by colony PCR using M13 universal primers. PCR products were purified (UltraClean™ PCR Clean-Up Kit, MOBI Laboratories) and sequenced at the Center for Human Genome Studies, São Paulo, Brazil. Subsequently, the region corresponding to the T-DNA was removed and the remaining flanking sequences were analysed with the BLAST tool (blastx, searching protein database using a translated nucleotide query) against the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/). The sequences were also analysed for the presence of ORFs (Open Reading Frame) using the ORF Finder tool (http://www.ncbi.nlm.nih.gov/), aiming to identify all ORFs using the standard or alternative genetic codes.

Analysis of transformants by fluorescence microscopy (MOF)

Transformants were randomly picked up and analysed for expression of the *gfp* gene, using fluorescence microscopy. For visualization of fluorescence in conidia, transformants were cultured at 28 °C for 15–25 d on PDA medium (containing 75 μg mL^{-1} hygromycin B). The CBS symptoms were cut with sterile scalpel and placed under sterile blades. The slides were prepared with sterile distilled water, sealed with colourless nail polish and directly analysed under a microscope. Analyses were performed using an epifluorescent microscope (Zeiss Axiophot-2) with a filter suitable for analysis of gfp. The samples were excited using a light with 490 nm wavelengths and the images were captured with a video camera attached to the microscope, using the ISIS software (Meta Systems, Germany). Inoculation of spores of *Guignardia mangiferae*, endophytic specie which has no ability to induce CBS symptoms, was used as a negative control (Fig 5C).

Mitotic stability of transformants

To determine the mitotic stability of the transformants, transformants were successively cultured on PDA without hygromycin B for ten generations, by subculture of hyphal tip and mycelium observation under epifluorescence microscope. Stable transformants were those which, after five successive passages in PDA medium, were able to grow on PDA amended with a concentration of hygromycin B previously determinate to this fungus, and retained the gfp fluorescence. Growth of *Guignardia citricarpa* 12P was successfully inhibited on PDA medium amended with hygromycin B (50 μg mL^{-1}), allowing the use of this antibiotic as a selective marker. Here we used a selective culture media containing 75 μg mL^{-1} hygromycin B, to give more reliability of the experiment.

Interaction between Guignardia citricarpa *and citrus fruit*

Aiming to study the ability of *G. citricarpa* transformants to colonize the tissues of citrus fruit, we used microscopic analysis. This analysis was conducted with both the wild type strain and transformed strain expressing gfp.

First of all, spores of *G. citricarpa* wild type and transformants were obtained (Fig 1). Leaves of oranges trees (*Citrus sinensis* 'Valência') were detached, washed, and cut in small fragments of 1 cm^2 and autoclaved. Eight fragments per plate were placed on the agar 3 % medium. Mycelia of *G. citricarpa* were inoculated adjacent to the leaves' fragments (Fig 1A). The plates were cultivated at room temperature (25 °C) with 12 h of photoperiod for 21 d. These asexual spores were inoculated in injuries done at the orange fruits by a sterilized stick and covered by moist cotton. The fruits were stored in a moist chamber (a box with humidity conditions, at room temperature) by 15 d until the symptoms appeared. The symptoms on fruits (black spots) were cut and placed on sterilized glass slides. The slides were prepared with sterile distilled water, sealed with colourless nail polish, and directly analysed under a microscope.

Physiological characterization of Guignardia citricarpa *transformants*

Aiming to check if the genetic insertion affected the ability of enzymes production of transformants, 15 randomly transformants were tested for their capacity to produce hydrolytic enzymes on solid medium (Hankin & Anagnostakis 1975). The enzymes analysed were protease, lipase, esterase, and pectinase.

Results

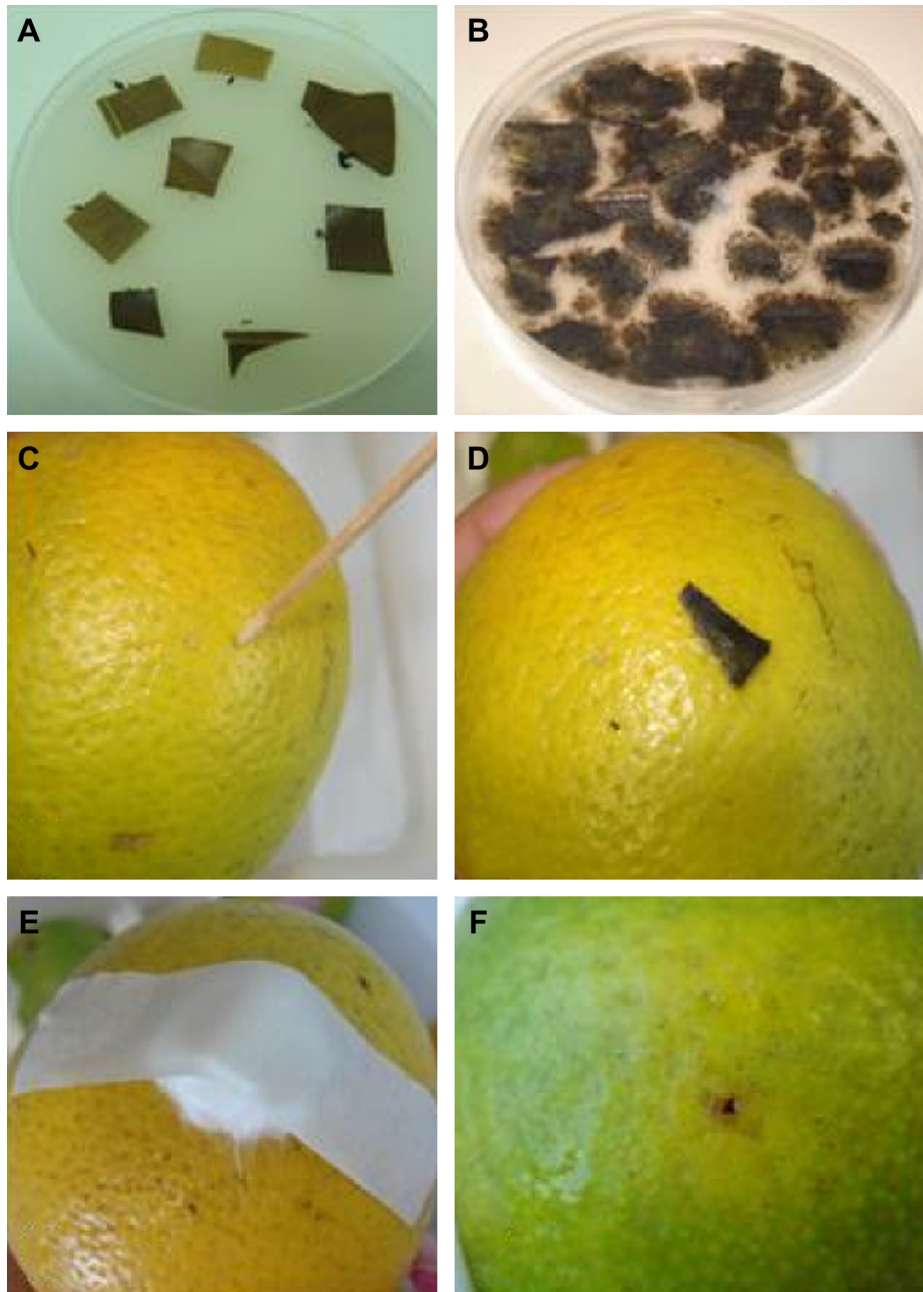### ATMT *of Guignardia citricarpa*

The results showed that *G. citricarpa* transformants were obtained in all ATMT evaluated conditions (Table 1). We observed that the better transformation efficiency condition (greater number of transformants per experiment) was obtained at 96 h for *G. citricarpa*–*Agrobacterium tumefaciens* cocultivation on a medium containing 200 mM AS, and a cellulose ester membrane (Millipore, 0.45 μm in porosity).

A solid substrate allows *G. citricarpa* and *A. tumefaciens* to grow close each other, so that the T-DNA may be transferred. We used three filter types as substrate and we observed that cellulose ester filter condition was better, showing larger number of transformants − 37 from 86 total; in the other hand, nylon filter showed a lower amount of transformants − 26 from 86 (Table 1). Although the presence of AS during *A. tumefaciens*–*G. citricarpa* cocultivation was imperative for transformation, the inclusion of 200 μM mL^{-1} generated 49 transformants per test, while 400 μM mL^{-1} resulted in 25 % less transformants. Transformation efficiency increased consistently as the cocultivation period increased, resulting in twofold more transformants when cocultivation time was extended from 48 h to 96 h at 28 °C (Table 1).

Fig 4 shows the appearance of transformation signs 60 d after transferring the membranes to selective medium containing hygromycin B.

The mitotic stability results show that 82 % of the transformants retained the phenotype of resistance to hygromycin B and expressed the gfp.

For Southern blotting analysis, the results showed that one transformant harboured a single copy of T-DNA integrated

Fig 1 – Methodology used to obtain *Guignardia citricarpa* spores and bioassay on citrus fruits. (A, B): Production of *G. citricarpa* spores on orange leaves in a petri dish, after 21 d (B); (C–E): mode of inoculation to perform the pathogenicity bioassay; (F): characteristic symptom of CBS on citrus fruits 15 d after the inoculation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

randomly in the host genome (Fig 2, L5). Also, two other analysed transformants presented two, three or more insertions (Fig 2 L3 and 4) in the genome.

Analysis of T-DNA flanking sequences

TAIL-PCR was used to identify the genomic DNA flanking the T-DNA insertion site (Liu & Whittier 1995). Using random and specific primers, and three amplification rounds, we successfully amplified the junction DNA of eight transformants. The LB sequences obtained by TAIL-PCR generally ranged from 200 to 300 bp. ORF were identified by BLAST tools. Among the eight T-DNA insertions analysed in these mutants, five were identified as the pFAT-gfp vector, and three were ORFs showed high homology (E ≤ 1e−65) to hypothetical fungal proteins with unknown function. However, using BLAST match (E ≤ 1e−5) in the National Center for Biotechnology Information (NCBI) nonredundant (nr) protein database, the sequences were

Table 1 — *Guignardia citricarpa* genetic transformation efficiency under various parameters tested: membrane type; concentration of AS; cocultivation time.

Parameter	Condition	Number of transformants in 20 plates
Membrane	Cellulose ester	37
	Paper filter	33
	Nylon	26
Concentration of AS ($\mu M\ mL^{-1}$)	200	49
	400	37
Cocultivation time (h)	48	30
	96	56
Total		86

similar to adenylyl sulphate reductase (CAP03145.1) of *Pseudomonas* sp. (Gama-proteobacteria), methyl-coenzyme M reductase (CBF64720.1) of *Sclerolinum contortum* (Annelida: Polychaeta), and N-acetylglucosaminyl-transferase (CAD90583.1) of *Bradyrhizobium* sp. (Alpha-proteobacteria).

GFP expression and characterization of ATMT transformants

Guignardia citricarpa mycelium was targeted with *gfp* gene under the control of the *gpd* promoter from *Aspergillus nidulans*. Following ATMT, 12 independent transformants were selected
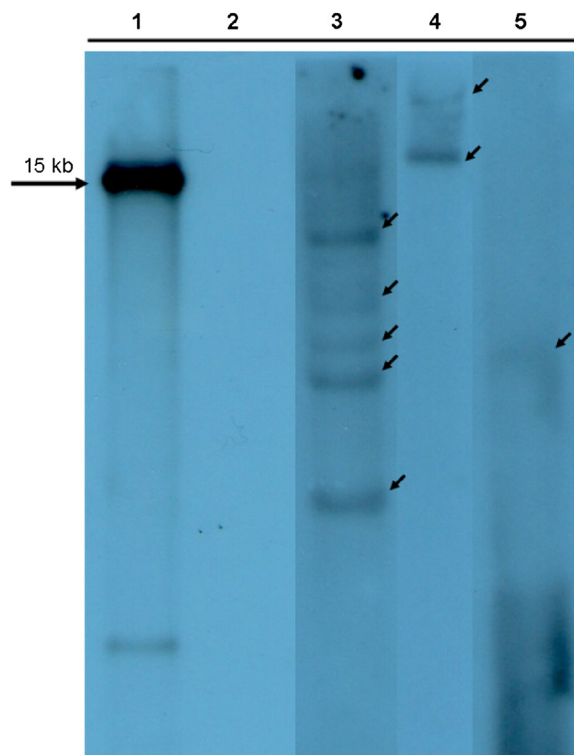
Fig 2 — Southern blot hybridization of three *Guignardia citricarpa* transformants and of *G. citricarpa* wild type 12P. Line 1 contains the vector pFAT-gfp (positive control); line 2 shows the wild type 12P *G. citricarpa* (negative control); lines 3–5 show the transformed isolates, chosen randomly. Arrows indicate the visualized bands.

for further analysis. All transformants were subcultured for five generations in PDA + HygB selective and nonselective medium, where all transformants displayed normal growth and were mitotically stable. Epifluorescence analysis showed that, as expected, the wild type *G. citricarpa* did not show any fluorescence under the excitation and detection conditions. All evaluated transformants exhibited diffuse cellular gfp localization, although punctuated hyphae fluorescence was also observed, suggesting the presence of protein aggregates or vacuolar accumulation of gfp (Fig 3). Additionally, green fluorescence was sometimes attenuated by, probably, dark pigments of *G. citricarpa*.

Guignardia citricarpa–citrus interaction

Spores of *G. citricarpa* wild 12P and transformants were inoculated on citrus fruits gave typical CBS symptoms. The injury caused by the fungi was characterized by round spots when skin becomes reddish (Fig 6) until finally becomes black. Transformant spores were inoculated on fruits and typical symptoms we observed (Fig 6). The behaviour of some 12P:gfp transformants was monitored by MOF during fruit infection (Fig 7). This is the first time that results using gfp were observed in *G. citricarpa*, showing the behaviour of the fungus. A dense hyphal mass involving mainly the intercellular spaces of the first layers of epidermal cells of plant tissue was observed (Fig 7).

All inoculated transformants induced typical CBS symptoms. The negative control, *Guignardia mangiferae*, showed no ability to induce CBS symptoms (Fig 5C).

Overall, the results (Fig 7) showed that the fungus colonized the fruit surface, forming a dense mycelial mass around the epidermal cells (Fig 7G–J). Furthermore, the analysis of cross sections revealed that all transformants of *G. citricarpa* were able to penetrate the host tissue and colonize the intercellular space of the first layers of the fruit peel epidermis (Fig 7K).

Moreover, the mycelial mass of transformants emits an intense green fluorescence (Fig 7I), allowing observation of fungal colonization over the plant tissue surface. It appears that first, the fungus colonizes the intercellular spaces of plant tissue (Fig 7J), starting with some body penetration, which is the early stage in the cell penetration (Fig 7K).

Physiological characterization of Guignardia citricarpa transformants

All *G. citricarpa* transformants (144) exhibited no morphophysiological (growth rate, colony morphology, and colour) changes when compared to wild type on PDA medium at 28 °C (Fig 4D and F). For physiological analysis, 15 transformants were tested for their capacity to produce hydrolytic enzymes on solid medium. The secretion of protease, lipase, and esterase did not differ between the wild type strain and the transformants (results not shown).

However, for pectinases production, it was observed significant differences between the wild type and some transformants (Fig 5). From 15 random transformants evaluated, five were similar to wild type 12P, while ten produced significantly lower amounts of pectinase.
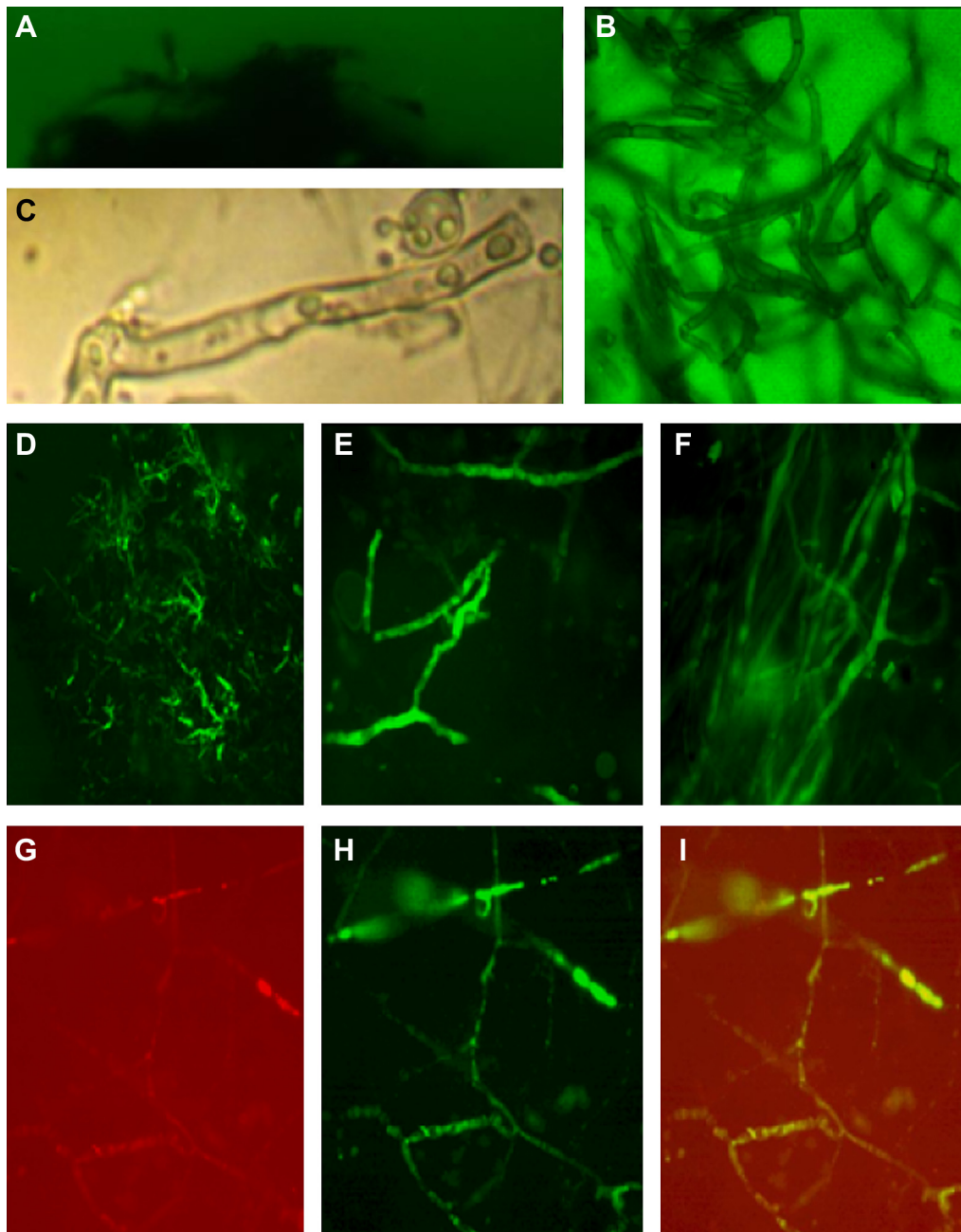
Fig 3 – The microscopic visualization of insertion of the gene for GFP from pFAT-gfp vector into the genome of *Guignardia citricarpa* transformants by epifluorescence optical microscopy. (A) Negative control, wild type 12P, mycelium without fluorescence (200×), (B) negative control, wild type 12P, hyphae without fluorescence (400×), (C) wild type 12P under optical microscope appearance of hyphae (400×) (D, E) *G. citricarpa* transformant mycelium exhibiting fluorescence (D: 100×, E: 400×), (F) *G. citricarpa* transformant grown in liquid medium with mycelia showing fluorescence in liquid medium (400×), (G–I) *G. citricarpa* transformant under different types of light filters: red (G) and green (H) and with the overlay of both. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Discussion

Based on previous reports of *Agrobacterium*-mediated transformation in fungal species (Mullins & Kang 2001; Sugui *et al.* 2005; Figueiredo *et al.* 2010), we adapted and optimized an ATMT system for gene transfer and random mutagenesis in *Guignardia citricarpa*, by using *hph* and *gfp* genes as the selectable and reporter markers, respectively. Moreover, this is the first time that results using gfp as gene reporter were observed in *G. citricarpa* transformants, allowing to observe the behaviour of the fungus growing pattern in the epidermis of the citrus fruits.

According to our data, the best ATMT conditions for random insertion of T-DNA in *G. citricarpa* genome were obtained with mycelium-*Agrobacterium tumefaciens* cocultivation for 96 h on cellulose ester as filter (Millipore, 0.45 μm in porosity)
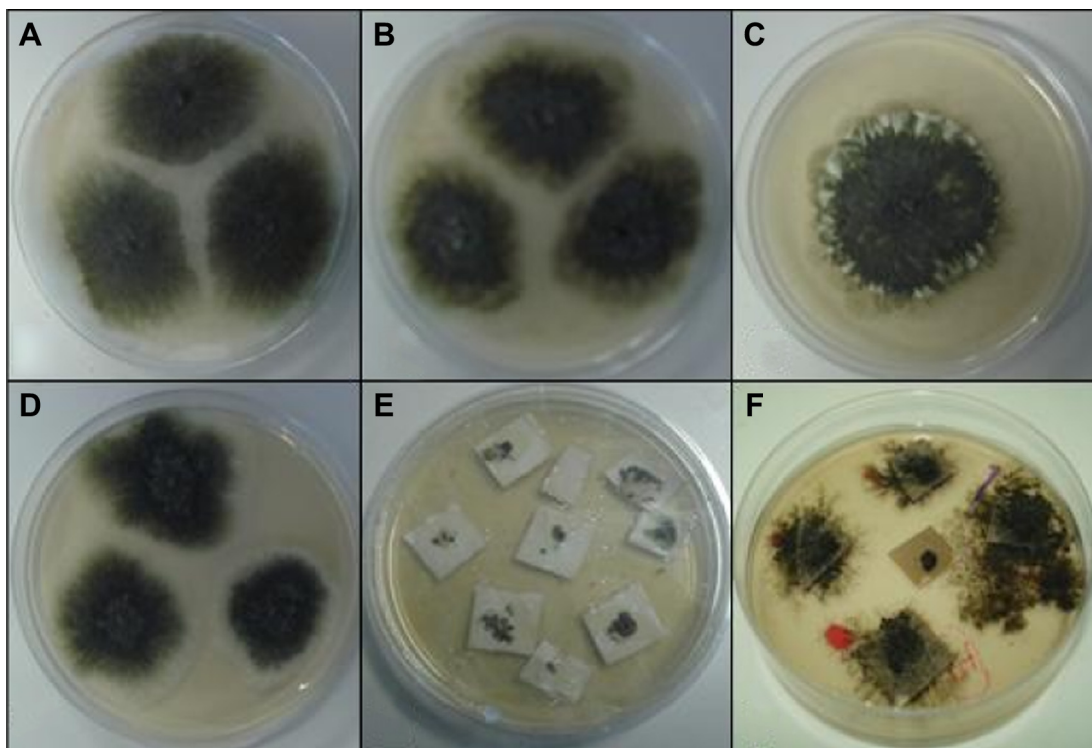
Fig 4 – *Guignardia citricarpa* colony aspect growing under different membrane types: (A) – filter paper; (B) – nylon; (C) – cellulose ester; (D) – *G. citricarpa* wild type 12P; (E) – agrotransformation experiment, cocultivation step; (F) – growing of *G. citricarpa* transformants hygromycin B resistant. No morphological differences of any of cultivation variants between transformants and wild type 12P.

with 200 µM mL^{-1} AS and transferred to culture medium for selection and growth for 60 d at 28 °C (Table 1). Under these conditions, in three independent experiments, the number of hygromycin-resistant transformant colonies per experiment ranged from 42 to 86, resulting in a total of 175 hygromycin B resistant transformants expressing gfp, which were picked and stored in a library with 144 transformants for future studies, especially for pathogenicity.

The establishment of the *Agrobacterium* transformation system for *G. citricarpa* enables the generation of transformants and use of this tool for studies of insertional mutagenesis

and gene disruption in order to identify important genes, such as those involved in the pathogenic mechanisms used by this fungus.

Different membranes such as nitrocellulose, nylon (Hybond), cellophane, and tissue paper have been used in the step of cocultivation for different protocols. In general, membranes of nitrocellulose and Hybond C have provided lower efficiencies of transformation (Vijn & Govers 2003; Michielse *et al.* 2005) than filter paper and Hybond N$^+$ (Zeilinger 2004). Fávaro (2009) observed that significant increases in the number of resistant colonies were obtained when nylon membranes
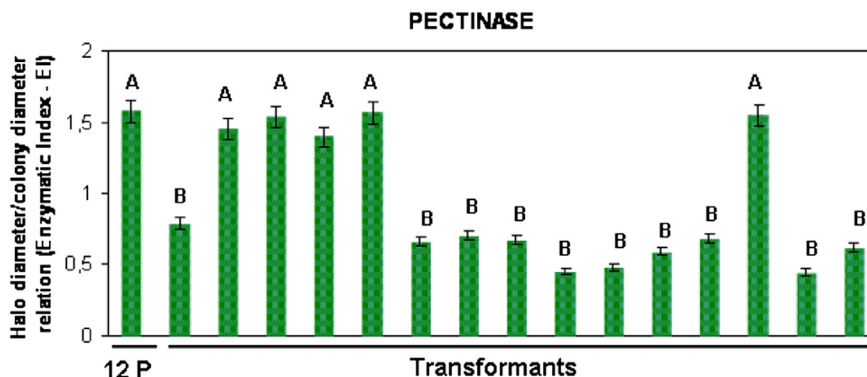
Fig 5 – Pectinase production profile by *Guignardia citricarpa* transformants. Mean values, with three replicates. Means followed by same letter do not differ by Tukey test (alpha = 0.05).
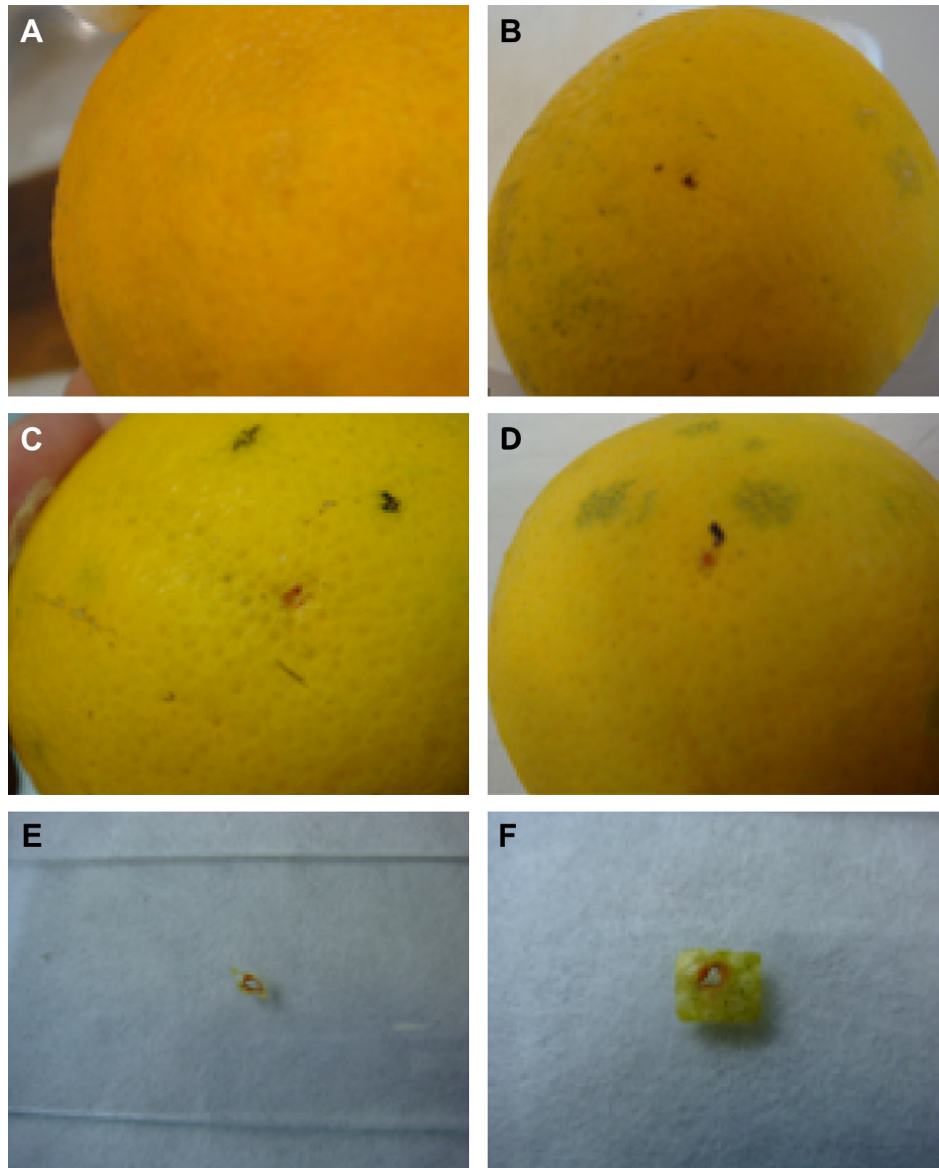
Fig 6 – Pathogenicity bioassay showing the ability of *Guignardia citricarpa* transformants to produce typical symptoms of CBS disease. Citrus inoculated with *G. citricarpa*. (A) Negative control, only wound the bark, (B) negative control, wound inoculated with spores of the endophytic species *G. mangiferae*, showing no symptoms developed, as expected, (C) positive control, spores of pathogenic specie *G. citricarpa* strain resulting in formation of the characteristic symptom of CBS disease, (D) characteristic symptom of the disease, caused *G. citricarpa* transformants, and (E, F) characteristic symptom of the disease, caused by inoculation of spores of *G. citricarpa* transformants. Observation in epifluorescence microscopy, cuts under sterile blades. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were used in coculture. The same was also reported for *Aspergillus fumigatus* (Sugui *et al.* 2005) and for the oomycete *Phytophthora infestans* (Vijn & Govers 2003).

Changes in cocultivation time have also caused variation in the transformation efficiency. For most fungal species, cocultivation time during 48 h resulted in a higher transformation efficiency (De Groot *et al.* 1998; Mullins & Kang 2001; Rho *et al.* 2001; Leal *et al.* 2004; Weld *et al.* 2006; Zhong *et al.* 2007), however, in some cases the extent of time can result in a greater number of transformants (Combier *et al.* 2003), as observed in this study (Table 1) and other fungi such as *Fusarium oxysporum*

(Mullins & Kang 2001), *Beauveria bassiana* (Leclerque *et al.* 2004), *Colletotrichum graminicola* (Flowers & Vaillancourt 2005), *Colletotrichum falcatum*, and *Colletotrichum acutatum* (Maruthachalam *et al.* 2008), *Cryptococcus neoformans* (McClelland *et al.* 2005), *Magnaporthe oryzae* (Betts *et al.* 2007), and *Epicoccum nigrum* (Fávaro 2009). *G. citricarpa* has a highly pigmented mycelial mass and a thick wall, factors that can hinder the transformation process, as was shown for *Coniothyrium minitans* (Rogers *et al.* 2004) and *E. nigrum* (Fávaro 2009).

Following this optimized protocol, a total of three experiments were conducted allowing the recovery of 175
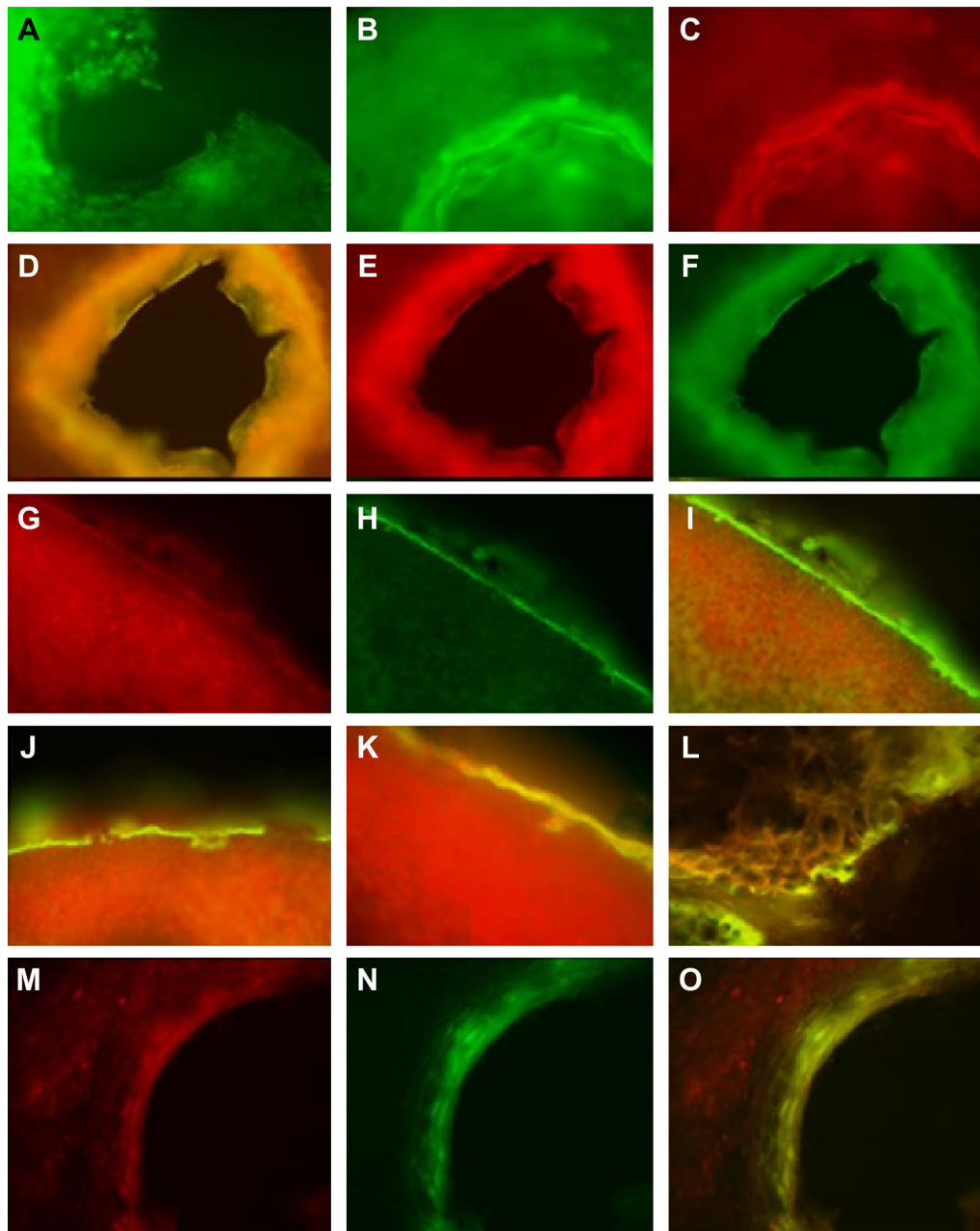
Fig 7 – Images of CBS symptoms caused by *Guignardia citricarpa* transformants under epifluorescence microscopy. The symptoms evaluated come from the fruit used in the pathogenicity test. (A–C) Negative control, damage caused by *Guignardia citricarpa* wild type 12P. Note that no fluorescence under the FITC filter (A, B) was observed, only a slight fluorescence coming from the plant tissue, collected under the Rhodamine filter (C) (100×), (D–F) negative control, fruit skin injury without infection of *G. citricarpa* transformants. Observe that there is no fluorescence being issued, even with the overlap of filters (D), FITC (F), and Rhodamine (E) (100×), (G–I) there is a mass of mycelium of a *G. citricarpa* transformant growing across the surface of plant tissue, emitting intense fluorescence (200×), (J) initial colonization of the intercellular spaces of plant tissue (200×), (K) note the formation of a cellular penetration organ, as an appressorium or a haustorium (200×), (L) detail of plant cells being enveloped by the transgenic fungi mycelial mass (100×); (M–O) details of the injury and colonization of the first layer of plant cells (100×). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hygromycin B resistant transformants expressing gfp. Although for most of fungi, such as *E. nigrum* (Fávaro 2009) and *Trichoderma virens* (Romão 2010), the transformants were obtained after a short period of time (4–15 d), *G.* *citricarpa* transformants were obtained only after 60 d. This is especially because *G. citricarpa* is a fungus that presents a slow growing, normally about 15 d in Petri dishes conditions.

Moreover, the number of transformants also differs significantly from the results found in literature, for example, in *Venturia inaequalis* they have results of 223 transformants per 200 mg of mycelium (Fitzerland *et al.* 2003) and *Verticillium fungicola* with 4.07 transformants per 10^5 conidia (Amey *et al.* 2002).

This is also due to the fact that *G. citricarpa Agrobacterium*-mediated transformants was used with mycelium as starting material for transformation. Other fungi with most voracious growth naturally sporulate under laboratory conditions. For this reason, we used mycelia to this study. Thus, spores or conidia can be used as starting material for transformation, which substantially increases the number of transformants generated. Generally, the increase in fungal cells leads to an increase in the number of transformants (Zwiers & De Waard 2001; Rolland *et al.* 2003; Reis *et al.* 2004; Rogers *et al.* 2004; Zeilinger 2004). It was also observed by Mikosh *et al.* (2001), demonstrating that the use of germinated spores, instead of mycelium, led to increased formation of transformants containing multiple copies of T-DNA.

G. citricarpa transformants presented high mitotic stability (82 %). High mitotic rate stability has also been described for different fungal species transformed with *gfp* gene by the *Agrobacterium* system (Fitzerland *et al.* 2003; Gorfer *et al.* 2007; Martino *et al.* 2007; Wang *et al.* 2008). There was also a high rate of sector formation in the transformants. The sector formation in *G. citricarpa* may be related to loss of RNAdf (Kava-Cordeiro *et al.* 2008).

Randomly selected transformants were analysed by epifluorescence microscopy (MOF) (Fig 3). The fluorescence emission was observed in the hyphae of all transformants evaluated, showing the stability of these transformants, resulted from stable mRNA synthesis and its translation. As expected, hyphae of wild type 12P *G. citricarpa* fluorescence were not detected. The expression of gfp in the transformants was distributed throughout the hyphae, which can be noted in some segments of hyphae with reduced expression or no expression of gfp (Fig 3). In *Leptosphaeria maculans* (Eckert *et al.* 2005), this pattern of gfp expression was reported, where the light microscopic analysis of the hyphae segments without expression revealed that these regions had collapsed cells in older hyphae. The non-uniform distribution of fluorescence in hyphae of some fungal species has been related to the exclusion of gfp vacuoles and mitochondria along with abundant accumulation of this protein in the nuclei and cytoplasm (Fernández-Ábalos *et al.* 1998).

In the results of this work, even a transgenic strain showing less production of pectinase, an important enzyme in pathogenicity systems (Jones *et al.* 1972; Isshiki *et al.* 2001), this lower production by itself was not sufficient to decrease the ability of this transformant to cause the CBS symptoms in fruits.

The different steps involved in differentiation and development of plant pathogens in the environment are still poorly known, showing more study is needed to assess the plant−fungus interaction.

The ATMT method is very attractive to obtain phenotypes mutants and identify genes related to these *via* random insertional mutagenesis. Responsive gene related to the pathogenic character of fungi species, could be identified or knocked out by this technique, allowing the study of several pathogenic systems (Rho *et al.* 2001; Combier *et al.* 2003; Leclerque *et al.* 2004; Rogers *et al.* 2004; Walton *et al.* 2005; White & Chen 2007; Zhong *et al.* 2007; Talhinhas *et al.* 2008). The establishment of this methodology for *G. citricarpa* future experiments can be efficiently performed in order to achieve similar results.

Overall, the results showed that the transformed fungus intensely colonizes the fruit surface, forming a dense mycelial mass around the epidermal cells. This is the first time that results using gfp were observed in *G. citricarpa*. Furthermore, the analysis of cross sections also revealed that *G. citricarpa* is able to penetrate the host tissue and colonize the intercellular space of the first layers of fruit peel epidermis. In this way, we can infer that the genetic transformation did not affect this ability to cause injury, i.e., as the insertion of exogenous DNA is random in the fungus genome, occurred at loci that have no effect on the capability of this fungus to develop disease symptoms.

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