MOLECULAR DIAGNOSIS OF Guignardia citricarpa IN ASYMPTOMATIC SWEET ORANGE TISSUE

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RESUMO – A pinta preta ou mancha preta dos citros, causada pelo fungo Guignardia citricarpa, é considerada uma doença quarentenária, que impõe restrições ao transporte de frutas frescas para países da União Europeia. A ocorrência de infecções latentes e o tempo para o diagnóstico por métodos convencionais levam à necessidade de validar protocolos moleculares rápidos, eficientes e reprodutíveis para detecção do patógeno em tecidos assintomáticos. Assim, este trabalho visou detectar G. citricarpa em tecidos de frutos sintomáticos e em folhas assintomáticas de laranja Pêra por PCR convencional e por PCR em tempo real. A especificidade e o limiar de detecção foram avaliados em amostras de tecidos de lesões em frutos e em folhas assintomáticas. Em folhas assintomáticas a presença do fungo foi detectada em baixas concentrações, nessas condições, a PCR em tempo real demonstrou ser viável, reprodutível e altamente sensível para a detecção do patógeno.

Termos para indexação: Citricultura, diagnose molecular, Phyllosticta citricarpa.
is interrupted by fruit maturation and climate conditions that favor the pathogen. The occurrence of long periods of latent infection demonstrates the importance of early detection, before symptoms emerge (HU et al., 2014).

In addition to being performed after symptom emergence, morphological diagnosis is further hampered by the similarity between G. citricarpa lesions and those caused by other pathogens, such as Alternaria alternata f. sp. citri and Diaporthe citri. The existence of an endophytic species (G. mangiferae) that is very similar to the pathogen were obtained (BAAYEN et al., 2002; WANG et al., 2012; HU et al., 2014) confirm the need for specific and efficient early diagnosis of the disease.

In addition to contributing to plant health, early detection of the pathogen in asymptomatic tissue is essential for the certification of citrus fruits destined for export to countries with stringent phytosanitary legislation. Molecular diagnosis stands out in the early and accurate detection of G. citricarpa as a low-cost technique with high specificity, sensitivity and reproducibility. This allows it to meet the high demand for sample processing and comply with the standards of International Plant Protection Organizations for the diagnosis of quarantine diseases, particularly G. citricarpa (EFSA, 2014).

Since there are no reports of techniques to detect latent G. citricarpa infection, and given the need for efficient and accurate diagnosis of CBR regardless of phenological growth stage, this study aimed to achieve early diagnosis of G. citricarpa in asymptomatic sweet orange leaves using conventional and real-time PCR.

**MATERIAL AND METHODS**

**Pathogen isolation and sample preparation**
Isolates of G. citricarpa were obtained from fruit with typical symptoms of the disease, on 16-year-old sweet orange (C. sinensis) plants collected in a commercial orange grove in the municipality of Pirenópolis, Goiás state (GO), Brazil. The pathogen was identified by analyses and morphological indicators and kept in oatmeal agar medium (BAAYEN et al., 2002).

Fruits with citrus black spot lesions were collected from 20 plants, totaling 20 oranges. Of these, ten were washed using detergent and rinsed with distilled water, and ten were used without being cleaned. The samples consisted of 250 mg tissue fragments from lesions on washed or unwashed fruit, as well as samples from a single lesion (measuring about 2 to 4 mm). All the samples were placed in 2 mL microtubes and stored in a freezer at -20 °C for subsequent DNA extraction.

Concomitantly, asymptomatic leaves were collected from orchards on two properties in the municipalities of Pirenópolis and Inhumas (GO). In Pirenópolis, samples were collected from a 40-hectare orchard of 16-year-old sweet orange plants (C. sinensis). Symptoms were observed on mature fruit concentrated in an 8-ha area of the orchard. Sampling was carried out in a 32-ha area that showed no visible symptoms of CBS. In Inhumas, sampling was performed in a 6.5-ha area containing 15-year-old sweet orange trees. No CBS symptoms were observed on the plot during sampling.

Asymptomatic leaves were randomly collected from 20 plants on each property. Eight leaves were collected from each plant at three different heights (lower, middle and upper third), totaling 24 leaves per plant. Two 0.5-cm wide sections were removed from each set of 8 leaves, one from each side of the midrib, and cut into smaller evenly-sized fragments. The working samples consisted of 250 mg of fragments, totaling 60 samples, which were stored at -20 °C until DNA extraction.

**DNA extraction**
The DNA extraction protocol was modified from the CTAB method and optimized at the Plant Diagnosis Laboratory (LDV) of the National Agricultural Laboratory in Goiás (LANAGRO-GO) (MORELLO, 2000). During the extraction process, reagent blanks (extraction controls) and environmental controls were used to demonstrate the lack of external nuclei acid contamination. In order to rupture the cell wall, 250 mg samples were placed in 2mL microtubes added with 1000 µL of 2% CTAB buffer \( \{ \text{p(CTAB)} = 20 \text{ g L}^{-1}, \text{c(NaCl)} = 1.4 \text{ mol L}^{-1}, \text{c(tris) = 0.1mol L}^{-1}, \text{c(Na2EDTA) = 0.02 mol L}^{-1}; \text{pH 8.0 adjusted with HCl} \} \), previously heated to 65 °C, and two glass beads were used to break the cell walls. The microtubes were agitated for 1 minute in a TissueLyser II bead mill at a maximum frequency of 30 oscillations per second. At the end of the process 20 µL of proteinase (20 mg mL\(^{-1}\)) were added and the microtubes were incubated in a water bath (65 °C) for one hour, with gentle agitation every ten minutes. The supernatant was transferred to a new microtube and added with 520 µL of a 24:1 (v/v) CIA solution (chloroform: isoamyl alcohol), with manual
inversion for 5 minutes.

For DNA precipitation, the samples were centrifuged at 12,000 g for ten minutes and 600 µL of the supernatant was transferred to nine 1.5 mL microtubes. Next, 300 µL of ammonium acetate (7.5 mol L \(^{-1}\)) and 600 µL of cold 96% isopropanol were added. After continuous, gentle manual inversion the solution was centrifuged at 12,000 g for 10 minutes and the supernatant was discarded. The precipitate was washed once with 500 µL of 70% ethanol, centrifuged again at 12,000 g for ten minutes and resuspended in 50 µL of TE (Tris = 10 mM; EDTA = 1 mM, pH 8.0). The microtubes were then added with 1 µL of RNase A (10 mg mL \(^{-1}\)) and incubated at 37°C ± 2°C for thirty minutes. The DNA samples were kept in a freezer at -20°C until quantification.

### Specificity analysis and limit of detection of *G. citricarpa* by conventional PCR

The protocol developed by Bonants et al. (2003) was used for specificity and limit of detection (LOD) testing in conventional PCR (EPPO/OEPP, 2009). The reaction conditions were: 0.60 µM of each primer, GcF3 (5’–AAA AAG CCG AGC CTA CCT TCA–3’) and GcR7 (5’–TGT CCG GCC AG–3’); 1x buffer (10x); 0.060 mM of dNTP Mix; 1.5 mM of MgCl\(_2\); 1 U of Taq DNA polymerase; 100 ng µL \(^{-1}\) of DNA; and type I water (nuclease-free) to obtain a final volume of 25 µL. Amplification was performed in a thermocycler (Eppendorf® Mastercycler ep Gradient), with initial denaturation at 95°C for ten minutes, 45 fifteen-second cycles at 95°C and one minute at 60°C.

To assess analytical specificity by real-time PCR, DNA from the same samples used in conventional PCR was submitted to reaction amplification. For LOD testing, an initial solution of 100 ng µL \(^{-1}\) of *G. citricarpa* DNA was submitted to seven serial dilutions with ten replicates.

### *G. citricarpa* detection in asymptomatic leaves

The detection of *G. citricarpa* in asymptomatic leaves was tested by comparing the LOD between conventional and real-time PCR, using the same DNA extraction protocol and the same methodology corresponding to each detection technique.

### RESULTS AND DISCUSSION

The DNA extracted from all the samples showed structural integrity, evident in the visualization of genomic DNA in 1% agarose gel and low polyphenol and polysaccharide concentrations, considering the A\(_{260}/A_{280}\) and A\(_{260}/A_{230}\) absorbance ratios (data not shown). The possibility of high quantity and quality DNA extraction from citrus fruit tissue using the method proposed here, with a view to detecting *G. citricarpa* by conventional and real-time PCR, is more practical, offers greater autonomy and reduces costs 7.5-fold on average in relation to commercial extraction kits (DEMEKE and JENKINS, 2010).

Analyses using specific molecular markers (GcF3 and GcR7) to identify the pathogen by conventional PCR amplified the 490 bp bands of the 16S ribosomal region of the positive controls. There was no amplification for DNA extracted from the other microorganisms or insect species, confirming the specificity of the technique against pathogens or...
pests that cause other diseases in citrus plants (Figure 1). As a result of the higher amount of fungal DNA extracted directly from the mycelium, the bands for these samples were more intense than those of the samples from the fruit lesions and the single lesion, a result also observed by Peres et al. (2007).

The amplification products of the 10 replicates of serial dilutions (1:10, 1:10², 1:10³ and 1:10⁴) obtained 100 % detection beginning at 100 ng µL⁻¹, 90 % at 10 ng and 70 % at concentrations of 1 and 0.1 ng visualized in agarose gel (1 %). Thus, conventional PCR was suitable for detecting G. citricarpa DNA with 90% repeatability of detection at a concentration of 10 ng and 70% at a minimum of 0.01 ng. No amplification was observed at 0.01 ng in agarose gel.

In real-time PCR analysis, the primers and probe used (GcF1 and GcR1/GcP1) were specific for G. citricarpa. The amplification products of the 10 replicates of serial dilutions (1:10, 1:10², 1:10³, 1:10⁴, 1:10⁵, 1:10⁶ and 1:10⁷), beginning with 100 ng of G. citricarpa mycelial DNA, produced amplifications for all the dilutions via real-time PCR, establishing the LOD with repeatability at a fungal DNA concentration of 10⁻⁵ ng (10 fg) (Table 1). Real-time PCR was suitable for detecting G. citricarpa DNA at a minimum concentration of 232 DNA copies, equivalent to 0.00001 ng of DNA. The number of copies was estimated as proposed by Hu et al. (2014). In addition to validating the in-house technique, these results indicate that the method is robust and more sensitive than conventional PCR, which may allow the phytopathogen to be detected in asymptomatic plant tissue, that is, before symptoms emerge.

For G. citricarpa detection using DNA extracted from asymptomatic leaves, conventional PCR was unable to amplify the expected products of the reaction for either the samples from Pirenópolis or Inhumas, totaling 120 samples (Table 2). These results differ from those obtained for the positive controls used in the reactions, which produced an amplicon for the GcF3/GcR7 marker. By contrast, in real-time PCR using the GcF1/GcR1 primers and GcP1 probe, G. citricarpa was identified in DNA from asymptomatic orange leaves collected at the two properties studied (Table 2).

Low levels of the fungus were recorded in asymptomatic leaves collected from areas with no CBS symptoms. This may explain the negative result in conventional PCR, which is inefficient at detecting the pathogen under these conditions, since it is less sensitive than real-time PCR. The highest detection frequency was observed in the municipality of Pirenópolis, where the presence of the pathogen on symptomatic fruit was confirmed in a specific plot on the property.

In this study, tests were conducted on asymptomatic leaves without inducing fungal growth prior to DNA extraction and amplification. This differs from research conducted by Meyer et al. (2012), who obtained positive results for G. citricarpa detection with conventional PCR, but induced emergence of the fungus on asymptomatic leaves by successively wetting and drying them over a period of four to ten days. Thus, the present study stands out for the specificity, sensitivity and speed of CBS detection prior to symptom emergence using real-time PCR for diagnosis in asymptomatic leaves, highlighting the importance of the technique in certifying the quality of citrus fruits and preserving disease-free areas.

The diagnosis of G. citricarpa in an asymptomatic orchard is highly beneficial in its prevention and control. It is important to note that although real-time PCR is highly sensitive, with an LOD of 0.00001 ng of DNA, 100 % detection was not achieved in the test samples, likely because some of the leaves collected showed no quiescent infection in the subcuticular mycelium. However, the detection frequency obtained was highly satisfactory for the identification of G. citricarpa in asymptomatic leaves, providing another alternative for preventive detection and diagnosis.

Identifying CBS infection prior to symptom emergence allows the application of more efficient control methods. Moreover, since G. citricarpa is a quarantine fungus subject to phytosanitary restrictions, real-time PCR can be used for preventive detection and as an additional option in CBS diagnosis to comply with regulations governing the exportation of fresh fruit to the European Union.
TABLE 1- Number of DNA copies and mean Ct observed for amplification of the specific *Guignardia citricarpa* region by real-time PCR at different DNA concentrations.

<table>
<thead>
<tr>
<th>DNA (ng µL⁻¹)</th>
<th>Positive/negative samples</th>
<th>Mean Ct</th>
<th>No. of copies*</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>10 / 0</td>
<td>22.18</td>
<td>232x10⁷</td>
</tr>
<tr>
<td>10</td>
<td>10 / 0</td>
<td>23.00</td>
<td>232x10⁶</td>
</tr>
<tr>
<td>1</td>
<td>10 / 0</td>
<td>25.70</td>
<td>232x10⁵</td>
</tr>
<tr>
<td>0.1</td>
<td>10 / 0</td>
<td>28.20</td>
<td>232x10⁴</td>
</tr>
<tr>
<td>0.01</td>
<td>10 / 0</td>
<td>30.36</td>
<td>232x10³</td>
</tr>
<tr>
<td>0.001</td>
<td>10 / 0</td>
<td>32.28</td>
<td>232x10²</td>
</tr>
<tr>
<td>0.0001</td>
<td>10 / 0</td>
<td>35.79</td>
<td>232x10¹</td>
</tr>
<tr>
<td>0.00001</td>
<td>10 / 0</td>
<td>39.51</td>
<td>232</td>
</tr>
</tbody>
</table>

* Number of copies estimated according to an average molecular weight for a DNA base pair of 4.3 x 10⁻⁶ pg (Hu et al., 2014).

TABLE 2- Detection frequency of *Guignardia citricarpa* by conventional and real-time PCR in asymptomatic leaves collected from the upper, middle and lower third of orange trees on two farms in the municipalities of Pirenópolis and Inhumas, Goiás state.

<table>
<thead>
<tr>
<th>Position</th>
<th>Pirenópolis</th>
<th>Inhumas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of samples</td>
<td>Conventional PCR (%)</td>
</tr>
<tr>
<td>Upper third</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Middle third</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Lower third</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Total/mean</td>
<td>60</td>
<td>0</td>
</tr>
</tbody>
</table>

FIGURE 1- *Guignardia citricarpa* detection by conventional PCR (primers GeF3 and GeR7), for specificity testing. 1) 1Kb Ladder; 2 and 3) DNA from a single lesion on washed fruit; 4 and 5) DNA from a single lesion on unwashed fruit; 6 and 7) DNA from lesions on washed fruit; 8 and 9) DNA from lesions on unwashed fruit; 10, 11, 12 and 13) DNA from *Guignardia citricarpa* isolates (mycelium); 14 and 15) DNA from *Alternaria* sp. isolate; 16) Blank.
CONCLUSIONS

Real-time PCR is efficient and applicable in the diagnosis of *G. citricarpa* in asymptomatic orange orchards, showing sensitivity for detection of the pathogen in leaf tissue with no visible symptoms. Given the proven specificity of the primers used, conventional PCR can be used to identify CBS in symptomatic fruits.

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REFERENCES


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