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# Enzymatic differences between the endophyte *Guignardia mangiferae* (Botryosphaeriaceae) and the citrus pathogen *G. citricarpa*

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**ABSTRACT.** The endophyte *Guignardia mangiferae* is closely related to *G. citricarpa*, the causal agent of citrus black spot; for many years these species had been confused with each other. The development of molecular analytical methods has allowed differentiation of the pathogen *G. citricarpa* from the endophyte *G. mangiferae*, but the physiological traits associated with pathogenicity were not described. We examined genetic and enzymatic characteristics of *Guignardia* spp strains; *G. citricarpa* produces significantly greater amounts of amylases, endoglucanases and pectinases, compared to *G. mangiferae*, suggesting that these enzymes could be key in the development of citrus black spot. Principal component analysis revealed pectinase production as the main enzymatic characteristic that distinguishes these *Guignardia* species. We quantified the activities of pectin lyase, pectin methylesterase and endopolygalacturonase; *G. citricarpa* and *G. mangiferae* were found to have significantly different pectin lyase and endopolygalacturonase activities. The pathogen *G. citricarpa* is more effective in pectin degradation. We concluded that

 

there are significant physiological differences between the species *G. citricarpa* and *G. mangiferae* that could be associated with differences in pathogenicity for citrus plants.

**Key words:** Citrus black spot; Fungal-plant interaction; Pectinase; Hydrolytic enzymes; Endophyte; Citrus pathogen

## INTRODUCTION

The endophyte *Guignardia mangiferae* A.J. Roy (anamorph: *Phyllosticta capitalensis*) has been confused with the citrus black spot pathogen *G. citricarpa* Kiely (anamorph: *Phyllosticta citricarpa* McAlpine) for many years. There are several reports of two morphologically similar species of *Guignardia* spp infecting citrus plants. These species differ with respect to their ability to cause citrus disease, growth in different culture media (Lee, 1969), and sequence of the internal transcribed spacer (ITS) (Meyer et al., 2001). Baayen et al. (2002) proved the occurrence of these two species in citrus, and described the endophytic one as *G. mangiferae* A.J. Roy. The development of molecular analytical methods has allowed differentiation of the citrus black spot pathogen from the endophytes (Bonants et al., 2003; Van Gent-Pelzer et al., 2007), but the physiological traits associated with pathogenicity have not yet been studied.

*G. mangiferae* has been reported as a ubiquitous endophyte and has been isolated from numerous plants (Baayen et al., 2002). *G. citricarpa* has a narrower host range, and has been isolated mostly from citrus species (Araújo et al., 2001; Glienke-Blanco et al., 2002; Durán et al., 2005). Besides the wider host range, the geographic distribution of *G. mangiferae* is also much wider than that of *G. citricarpa* and remarkably includes regions in which the disease caused by *G. citricarpa* has not been reported (Everett and Rees-George, 2006).

Any fungus that attempts to colonize a higher plant must contend with physical barriers of the host: surface waxes, cutin and the cell wall. One of the most conspicuous effects of microorganisms on the plant cell wall is enzymatic degradation (Walton, 1994) by the activity of fungal cell wall-degrading enzymes, which may facilitate fungal growth and provide the fungus with nutrients. In a number of systems, a strong correlation has been found between the presence of pectinolytic enzymes, disease symptoms, and virulence (Durrands and Cooper, 1988). Pectinases are a group of enzymes that catalyze degradation of the pectic polymers present in plant cell walls and render them more susceptible to further breakdown by other enzymes. Pectin-degrading enzymes have been reported as the first extracellular degradative enzymes produced during infection (Mankarios and Friend, 1980). Specifically, endopolygalacturonases (endoPGs) and pectin lyases (PL) have been proposed to have an important role in fungal pathogenicity (Wattad et al., 1995; Shieh et al., 1997; Ten Have et al., 1998; Rogers et al., 2000; Wagner et al., 2000; Yakoby et al., 2000; Garcia-Maceira et al., 2001; Basaran et al., 2007).

In this study, in order to understand the interaction between host plant and *Guignardia* spp, fungal strains were first classified as *G. mangiferae* or *G. citricarpa* by ITS1-5.8S-ITS2 sequence comparison. These species were then compared regarding production of amylases, cellulases and pectinases on solid medium, and pectinase activities were

quantified. The results showed that *G. mangiferae* and *G. citricarpa* have significantly different abilities to produce hydrolytic enzymes, especially the pectinases, which may be associated with the strategy used by these species to infect, colonize and induce or not disease symptoms in citrus host plants.

## MATERIAL AND METHODS

### Fungal isolates and culture methods

A total of 36 isolates of *Guignardia* spp were used in the present study; some were from the laboratory of Microbial Genetics (ESALQ/USP, Piracicaba, Brazil), and the origins of the others are given in Table 1. The isolates were grown on potato dextrose agar (PDA; Merck) at 28°C for 10-20 days.

### Molecular approach

To extract genomic DNA, isolates were grown on PD for 10 days at 28°C without shaking. The mycelia were collected by filtration and ground in liquid nitrogen, and 4 mL lysis buffer (200 mM Tris-HCl, pH 8.0, 250 mM NaCl, 25 mM EDTA, 1% SDS) was added for each gram of mycelium. The mixture was incubated for 15 min at 70°C, and DNA was purified by the standard phenol:chloroform method (Sambrook et al., 1989). The DNA was precipitated with 60% volume of isopropanol and pelleted by centrifugation at 14,000 *g* for 10 min. The DNA was washed with 70% ethanol, dried, and dissolved in TE buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, pH 8.0). DNA integrity was verified by electrophoresis on a 0.8% agarose gel.

The region of ribosomal DNA containing the ITS1-5.8S-ITS2 fragments was amplified by polymerase chain reaction (PCR) using the primers ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3'). PCRs were performed in a final volume of 50 µL containing 50 ng DNA template, 0.2 mM each dNTP, 0.2 µM each primer, 3.7 mM $MgCl_2$, 0.4 U *Taq* DNA polymerase (Invitrogen, Brazil), 50 mM KCl, and 20 mM Tris-HCl, pH 8.4. The reaction mixtures were incubated in a Perkin Elmer thermocycler with an initial denaturation step at 94°C for 5 min, and then 32 cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 72°C, followed by a final extension at 72°C for 7 min. The PCR products were purified with the UltraClean PCR Clean-up Kit (MoBio Laboratories Inc.) and sequenced using the ITS-4 primer at the University of Mogi das Cruzes, São Paulo, Brazil.

Nucleotide sequences were analyzed with BLAST (Altschul et al., 1990) and compared with representative *Guignardia* spp ITS sequences from GenBank (National Center for Biotechnology Information; NCBI), aligned and analyzed phylogenetically using the MEGA software version 3.1 (Kumar et al., 2004). Clustering was calculated by the neighbor-joining method with 1000 bootstrap replicates based on genetic distances calculated via the Jukes and Cantor model.

### Physiologic approach

Semi-quantitative tests were performed on solid medium to evaluate the abilities of

the fungi to produce amylases, endoglucanases (CMCase) and pectinases. For that, fragments of fungus grown on PDA (5-mm$^2$ plugs) for 10 days at 28°C were placed onto minimal medium (MM) (Pontecorvo, 1953) supplemented with 10 g starch, carboxymethyl cellulose or citric pectin. After 5 days of growth, the isolates were evaluated for pectinase and amylase (Hankin and Anagnostakis, 1975), and for endoglucanase (Teather and Wood, 1982). The enzymatic activity was estimated by the halo/colony diameter ratio, and three replicates were used in all analyses.

Quantitative tests were performed to evaluate the ability of *G. citricarpa* and *G. mangiferae* to degrade pectin. The enzymatic activities of PL, pectin methylesterase (PME), and endoPG were assessed by growing *G. mangiferae* (G1, G13, G16, 515, LRS36/99, 1F1, 1F2) and *G. citricarpa* (G8, G12, F22, L4-F6, 7LE10, LRS22/99, LRS34/98) for 5 days at 28°C in 30 mL liquid MM containing 10 g/L citric pectin. The cultures were harvested and the cell-free supernatant of three replicates was analyzed. The protein concentration of the extracts was measured by the Bradford method (1976).

PL activity was quantified as the increase in absorbance at 235 nm after incubation of the extract for 45 min at 45°C with 2.5% pectin followed by the addition of HCl to a final concentration of 0.01 M. A molar extinction coefficient of 5550 (Albersheim, 1966) was used to calculate the PL activity. The endoPG activity was measured as the release of reducing groups from 0.1% (w/v) polygalacturonic acid and quantified by the cyanoacetamide method (Gross, 1982). PME activity was determined by measuring the decrease in absorbance at 600 nm due to acid production during de-esterification of 4% pectin (Zamski and Peretz, 1996).

## Data analysis

The production of hydrolytic enzymes was subjected to principal component analysis (PCA) using the Canoco 4.5 software (Ter Braak and Šmilauer, 2002). The first two principal components were plotted to visualize the grouping of samples. The Monte Carlo statistical test with 499 random permutations was used to obtain the P values of the factors.

The enzymatic data were analyzed using the statistical package SAS© (1989-1996, SAS Institute Inc., Cary, USA). The enzymatic activity data were transformed using $(x + 0.5)^{1/2}$. All experiments were subjected to analysis of variance considering the homogeneity of variances. The Student *t*-test was used to compare the groups of isolates of *G. mangiferae* and *G. citricarpa*.

## RESULTS

The phylogeny based on the ITS1-5.8S-ITS2 sequences revealed the clustering of the 36 *Guignardia* spp strains into two major groups (A and B) (Table 1, Figure 1). Group A was composed of 21 isolates obtained from healthy tissues of different hosts, where the fungus had been present endophytically, as well as *G. mangiferae* GenBank sequences. Group B included 14 isolates that originated from fruits and leaves with classical black spot symptoms, one isolate from *Catharanthus roseus* stem and *G. citricarpa* GenBank sequences. Both groups A and B were highly similar only to *G. mangiferae* and *G. citricarpa*, respectively.

**Table 1.** Isolates of *Guignardia* spp evaluated in the present study and their species classification according to sequencing identification.

| Isolate | Host | Source | Origin | ITS1-5.8S-ITS2 sequencing identification |
|---|---|---|---|---|
| G1 | *Citrus sinensis* | Leaf | USA[a] | *Guignardia mangiferae* |
| G2 | *Citrus sinensis* | Leaf | USA[a] | *Guignardia mangiferae* |
| G4 | *Citrus sinensis* | Leaf | USA[a] | *Guignardia mangiferae* |
| G7 | *Citrus sinensis* | Leaf | USA[a] | *Guignardia mangiferae* |
| G11 | *Citrus sinensis* | Leaf | USA[a] | *Guignardia mangiferae* |
| G13 | *Citrus sinensis* | Leaf | USA[a] | *Guignardia mangiferae* |
| G14 | *Citrus sinensis* | Leaf | USA[a] | *Guignardia mangiferae* |
| G16 | *Citrus sinensis* | Leaf | USA[a] | *Guignardia mangiferae* |
| G17 | *Citrus sinensis* | Leaf | USA[a] | *Guignardia mangiferae* |
| G21 | *Citrus sinensis* | Leaf | USA[a] | *Guignardia mangiferae* |
| Ser | *Eucalyptus* spp | Branch | Brazil[e] | *Guignardia mangiferae* |
| 492 | *Citrus limon* | Leaf | Argentina[d] | *Guignardia mangiferae* |
| 61T | *Citrus limon* | Branch | Argentina[d] | *Guignardia mangiferae* |
| 63 | *Citrus limon* | Leaf | Argentina[d] | *Guignardia mangiferae* |
| 515 | *Citrus limon* | Leaf | Argentina[d] | *Guignardia mangiferae* |
| 7L | *Citrus sinensis* | Leaf | Brazil[b] | *Guignardia mangiferae* |
| LRS36/99 | *Citrus sinensis* | Fruit | Brazil[c] | *Guignardia mangiferae* |
| 41/99 | *Citrus reticulata* | Leaf | Brazil[b] | *Guignardia mangiferae* |
| 1F1 | *Catharanthus roseus* | Leaf | Brazil[e] | *Guignardia mangiferae* |
| 1F2 | *Catharanthus roseus* | Leaf | Brazil[e] | *Guignardia mangiferae* |
| 1F3 | *Catharanthus roseus* | Leaf | Brazil[e] | *Guignardia mangiferae* |
| G3 | *Citrus sinensis* | Leaf | USA[a] | *Guignardia citricarpa* |
| G6 | *Citrus sinensis* | Leaf | USA[a] | *Guignardia citricarpa* |
| G8 | *Citrus sinensis* | Leaf | USA[a] | *Guignardia citricarpa* |
| G9 | *Citrus sinensis* | Leaf | USA[a] | *Guignardia citricarpa* |
| G10 | *Citrus sinensis* | Leaf | USA[a] | *Guignardia citricarpa* |
| G12 | *Citrus sinensis* | Leaf | USA[a] | *Guignardia citricarpa* |
| LRS22/99 | *Citrus limon* | Leaf | Brazil[c] | *Guignardia citricarpa* |
| LRS25/98 | *Citrus sinensis* | Fruit | Brazil[c] | *Guignardia citricarpa* |
| LRS34/98 | *Citrus sinensis* | Fruit | Brazil[c] | *Guignardia citricarpa* |
| Crav1 | *Citrus* spp | Fruit | Brazil[c] | *Guignardia citricarpa* |
| Crav2 | *Citrus* spp | Fruit | Brazil[c] | *Guignardia citricarpa* |
| F22 | *Citrus sinensis* | Fruit | Brazil[b] | *Guignardia citricarpa* |
| L4-F6 | *Citrus sinensis* | Fruit | Brazil[b] | *Guignardia citricarpa* |
| 7LE10 | *Citrus sinensis* | Fruit | Brazil[b] | *Guignardia citricarpa* |
| 1C | *Catharanthus roseus* | Stem | Brazil[e] | *Guignardia citricarpa* |

Isolates supplied by: [a]George Carroll (Department of Biology, University of Oregon, Eugene, OR, USA); [b]Carlos I. Aguilar-Vildoso (Clínica Fitopatológica, Centro APTA Citros Sylvio Moreira - IAC, Cordeirópolis, SP, Brazil); [c]Fundecitrus; [d]Estela L. Durán (Cátedra de Fitopatologia, Facultad de Agronomía y Zootecnia, Universidad Nacional de Tucumán, Tucumán, Argentina); [e]Authors' culture collection.

The enzymatic semi-quantitative tests performed on solid medium showed that *G. citricarpa* produced significantly greater amounts of amylases (P = 0.0010), endoglucanases (P = 0.0077) and pectinases (P < 0.0001) than did *G. mangiferae* (Table 2). PCA (Figure 2), considering the production of hydrolytic enzymes, showed that pectinase production was the main factor discriminating the two species *G. citricarpa* and *G. mangiferae*.


**Figure 1.** Phylogram with bootstrap values (1000 replicates) derived from DNA sequence data of the ITS region (ITS1-5.8S-ITS2) for the 36 *Guignardia* strains studied and 13 sequences of *Guignardia* species from GenBank. Group A represents the *G. mangiferae* isolates and group B represents the *G. citricarpa* isolates. The *G. laricina* sequence was included as the outgroup.

**Table 2**. Production of hydrolytic enzymes by *Guignardia mangiferae* and *G. citricarpa* showing the differences, standard errors, and *t*-test results.

| Enzyme | Mean | | Estimate | Standard error | *t* | P [a] |
|---|---|---|---|---|---|---|
| | *G. mangiferae* | *G. citricarpa* | | | | |
| Amylase | 1.53 | 1.84 | -0.51 | 0.149 | -3.42 | 0.0010 |
| Endoglucanase | 1.18 | 1.42 | -0.32 | 0.115 | -2.74 | 0.0077 |
| Pectinase | 1.06 | 2.77 | -0.83 | 0.144 | -5.77 | <0.0001 |

[a]Nominal significance level in the *t*-test for comparison.

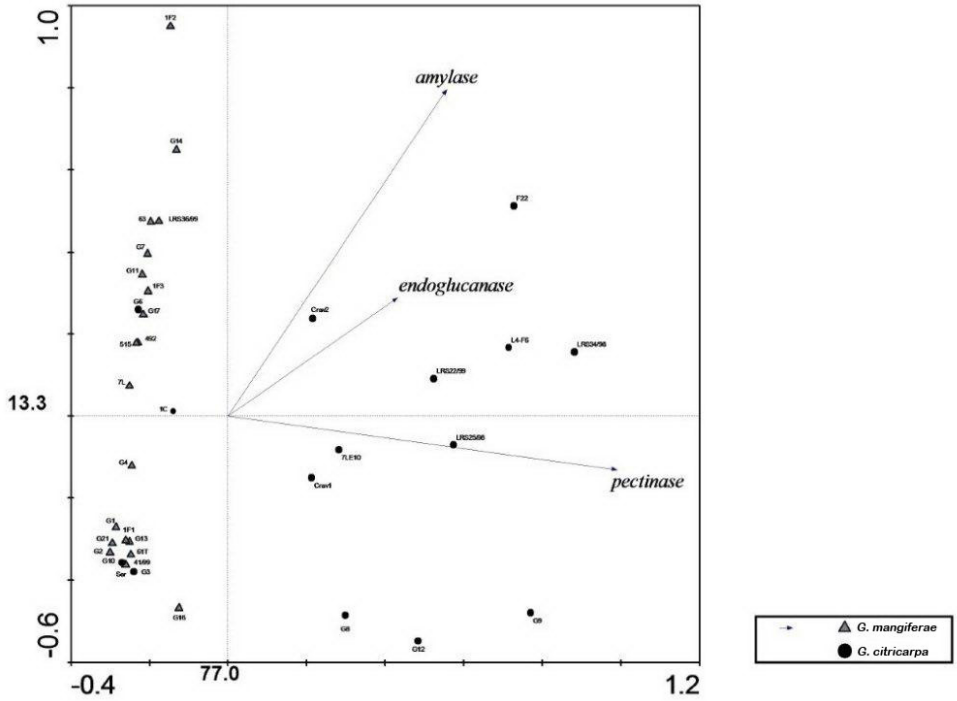


**Figure 2.** Principal component analysis (PCA) based on the production of hydrolytic enzymes (amylase, endoglucanase and pectinase) on solid media by *Guignardia mangiferae* and *G. citricarpa* isolates. Values in axes indicate the variance-explained axis.

The ability of the *Guignardia* spp to produce different pectinases was assessed on the basis of three activities: PL, PME and endoPG. The results showed that *G. citricarpa* and *G. mangiferae* had significantly different PL ($P < 0.0001$) and endoPG ($P = 0.0335$) activities, but no significant difference was observed for PME ($P = 0.1077$) activity (Table 3).

**Table 3.** Production of three pectinolytic enzymes (endopolygalacturonase, pectin methylesterase and pectin lyase) by *Guignardia mangiferae* and *G. citricarpa* showing the differences, standard errors, and *t*-test results.

| Pectinolytic enzyme | Mean | | Estimate | Standard error | *t* | P [a] |
|---|---|---|---|---|---|---|
| | *G. mangiferae* | *G. citricarpa* | | | | |
| Endopolygalacturonase [b] | 1.13 | 1.63 | 1.69 | 0.756 | 2.24 | 0.0335 |
| Pectin methylesterase [c] | 0.74 | 0.77 | -0.05 | 0.033 | -1.66 | 0.1077 |
| Pectin lyase [d] | 5.79 | 6.68 | -11.58 | 2.450 | -4.73 | <0.0001 |

[a]Nominal significance level in the *t*-test for comparison; [b]One unit of enzymatic activity was defined as the amount of enzyme required to release 1 µmol of reducing sugar groups per milliliter per minute; [c]One unit of enzyme activity was defined as the ratio between absorbance (600 nm) and total protein per minute; [d]One unit of enzyme activity was defined as the amount of enzyme that produced 1 nmol of unsaturated uronides per milliliter of culture extract per minute.

## DISCUSSION

There is an extensive literature describing the molecular and morphological differences that distinguish the two species of *Guignardia* that inhabit citrus plants (Meyer et al., 2001; Baayen et al., 2002; Bonants et al., 2003; Durán et al., 2005; Everett and Rees-George, 2006; Van Gent-Pelzer et al., 2007). However, there has been no report of the enzymatic aspects associated with species. In the present study, we evaluated the enzymatic profiles of *G. mangiferae* and *G. citricarpa*. The results showed that *G. citricarpa* strains produced significantly greater amounts of hydrolytic enzymes (Table 2) and that the main factor distinguishing the *Guignardia* species was the difference in the level of pectinase production (Figure 2). These data suggest that the level of pectinase production could be used to differentiate these two species. Further studies are required to establish the possible role of these enzymes in host-pathogen and host-endophyte interactions.

The differences in the level of production of pectinases suggest that these enzymes may have distinct roles during the interaction of the pathogen *G. citricarpa* and the endophyte *G. mangiferae* with citrus plants. *G. citricarpa* penetrates directly into citrus tissues, forms subcuticle mycelia and remains latent for 4-6 months (Timmer, 1999); after this period, injury is induced mainly in fruit, pectin-rich organs, showing that the pectinolytic enzymes are not involved primarily in either invasion or nutrition, but are related to disease symptoms. In the case of *G. mangiferae*, the pectinases may be important for endophytic colonization or even for the colonization of senescent and dead tissues. Interestingly, in the genus *Colletotrichum* a pectin lyase gene has been proposed to be fundamental to defining the fungus lifestyle, from endophytic mutualist to pathogen (Freeman and Rodriguez, 1993; Wattad et al., 1995; Redman et al., 1999; Yakoby et al., 2000).

The quantification of the enzymatic activity of three pectinases (PL, PME and endoPG) showed that *G. citricarpa* and *G. mangiferae* had significantly different levels of PL and endoPG activity, but similar levels of PME activity (Table 3). PL and endoPG depolymerize the pectin backbone, whereas PME alters the structure of the intact pectin backbone by catalyzing the hydrolysis of galacturonate methyl esters (Kester et al., 2000). This suggests that the lack of difference between *G. citricarpa* and *G. mangiferae* PME activity is probably due to the enzyme activity pattern; i.e., endoPG and PL act synergistically in the development of plant pathogenesis. The endoPGs are the first detectable enzymes secreted by phytopathogenic fungi when they are grown *in vitro* on plant cell walls and during the infection process (Johnston and Williamson, 1992). Evidence that polygalacturonases are involved directly in the pathogenic process has been provided by targeted mutagenesis in *Aspergillus flavus* (Shieh et al., 1997), *Botrytis cinerea* (Ten Have et al., 1998), *Fusarium oxysporum* (Garcia-Maceira et al., 2001), and *Penicillium olsonii* (Wagner et al., 2000). Likewise, Rogers et al. (2000) reported that the disruption of two PL genes drastically reduced the virulence of *Nectria haematococca* in peas (*Pisum sativum*). A long series of publications on production of lyase enzymes by pathogenic *vs* nonpathogenic *Colletotrichum* also showed that a single PL gene is essential for *C. magna* pathogenic abilities and is a pathogenicity factor required for the penetration and colonization of *Colletotrichum* species (Freeman and Rodriguez, 1993; Wattad et al., 1995; Redman et al., 1999; Yakoby et al., 2000). However, there are also reports on the disruption of endoPG and PL genes that show limited or no contribution to the pathogenic abilities of the pathogen (Bowen et al., 1995; Guo et al., 1995; Scott-Craig et al., 1990, 1998; Roncero et al., 2003).

The conflicting results of the influence of these enzymes in fungal pathogenicity demonstrate the difficulties encountered in investigating these interactions and behaviors. It is worth noting that the disruption of a certain gene may have no detectable effect, because other genes can mask its inactivity.

The results of this study showed that these two species of *Guignardia* secrete significantly different levels of hydrolytic enzymes, especially pectinases. Therefore, *G. citricarpa* and *G. mangiferae*, which are closely related but interact differently with the same host, can be distinguished by both genetic and physiological features. Further studies, such as gene inactivation, are required to improve the understanding of the interactions of these two *Guignardia* species in citrus plants, and may contribute to providing new approaches to citrus black spot control.

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