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Genetic variability in the endophytic fungus *Guignardia citricarpa* isolated from citrus plants

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Abstract

During some phases of of their life-cycle endophytic fungi colonize plants asymptomatically being found most frequently inside the aerial part of plant tissues. After surface disinfection of apparently healthy leaves from three varieties of mandarin orange and one tangor, and after incubation on appropriate culture medium, 407 fungal isolates were obtained, giving a total infection frequency of 81%. No fungal growth was observed from disinfected seeds, indicating that fungi are probably not transmitted via seeds. Of the fungal isolates, 27% belonged to the genus *Guignardia*, with 12 isolates being identified as *Guignardia citricarpa* Kiely, which is described as a citrus pathogen. The isolates were variable in respect to the presence of sexual structures and growth rates. Most of the isolates produces mature asci, supporting the hypothesis that they are nonpathogenic endophytes, which recently were identified as *G. mangiferae*. High intraspecific genetic variability (an average similarity coefficient of 0.6) was detected using random amplified polymorphic DNA (RAPD) markers generated by seven different primers. The highest similarity coefficient (0.9) was between isolates P15 and M86 and the smallest (0.22) between isolates P15 and C145. These results did not allow us to establish an association between genetic similarity of the fungal isolates and the citrus varieties from which they were obtained.

Key words: citrus, endophytes, genetic variability, *Guignardia citricarpa*, mandarin, RAPD.

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Introduction

Endophytic fungi asymptomatically colonize plant tissues during some phases in their life cycle (Petrini, 1991). Although the nature of their interaction with hosts is still not well understood, it has been established that in many cases the relationship can be symbiotic, antagonistic or even neutral. Some of these microorganisms are relatively easy to isolate as they grow in simple culture media and can be obtained after external disinfection of plant tissues. Investigations related to endophytic microorganisms isolated from several plants and their tropical hosts have recently increased, due to the increasing importance of fungi in biological control and the ongoing search for pharmacologically active compounds (Pereira *et al.*, 1993; Breen, 1994; Rodrigues, 1994; Rodrigues and Dias-Filho, 1996; Miles *et al.*, 1998; Strobel and Long, 1998; Pereira *et al.*, 1999; Azevedo *et al.*, 2000; Pinto *et al.*, 2000). Little is known about the microbial endophytic community of citrus (Araujo *et al.*, 2001) and the possible impact of endophytes on yield and, especially, on the control of citrus plant diseases, making the isolation and study of these microorganisms important for morphological, physiological and genetic studies.

The research reported in this paper was designed to isolate and characterize the endophytic fungal population of some *Citrus* species and showed that *Guignardia citricarpa* is one of the fungi most frequently present in citrus. Because of its importance in phytopathology and the present lack of genetic data, we also assessed the genetic diversity of *G. citricarpa* isolates using random amplified polymorphic DNA (RAPD) markers and cytological analysis.

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Materials and Methods

Fungal isolates

To isolate the endophytes, 85 apparently healthy leaves and 200 seeds were collected from three varieties of mandarin orange (varieties Poncã and Cravo of *Citrus reticulata* cv. Blanco and variety Mexerica of *C. deliciosa*

cv. Tenore and one tangor (variety Murcote, a hybrid of *C. reticulata* cv. Blanco and *C. sinensis* cv. Osbeck) growing at the Sylvio Moreira Citriculture Center, Agronomic Institute of Campinas, Cordeirópolis, São Paulo, Brazil. These plants were approximately 11 years old. Fungi were isolated using the method of Petrini (1986) *i.e.* leaves were surface sterilized by immersion in 70% (v/v) ethanol for 1 min, sodium hypochlorite 3% (v/v available chlorine) for 4 min and washed in 70% ethanol for 30 seconds, after which they were rinsed several times in sterilized distilled water and cut into five 5-7 mm fragments in a sterilized chamber and transferred in serial order to plates containing the complete culture medium (CM) of Pontecorvo *et al*. (1953). Tetracycline was added to the medium at 100 µg/mL to inhibit bacterial growth and the plates incubated at 28 °C for a maximum of 2 weeks. The seeds received similar treatment but half of the seeds used were cut apart before plating.

Isolates were purified, coded, and ,when possible, identified by standard mycological methods . *Guignardia citricarpa* isolates P15, P45, P210 (from Poncã), C145, C224, C320 (from Cravo), X68, X80, X171 (from Mexerica) and M86, M131, M216 (from Murcote) were subjected to morphological and RAPD analyses.

Identification and cytology

The isolates were cultured on CM, potato dextrose agar (PDA) and malt extract agar (MEA) as appropriate. Cytological identification and analysis of the isolates were carried out by observing the reproductive structures (sexual and asexual), using optical microscopy and the Giemsa staining technique of Furtado (1968), as modified by Luna (1985), and lactophenol (Onions *et al.*, 1981).

Preparation of DNA samples

Genomic DNA was obtained according to the methodology described by Raeder and Broda (1985), with modifications. Isolates were grown in agitated CM broth for 4 days at 28 °C. The resulting mycelia were filtered, ground in liquid nitrogen and dissolved in 4 mL of Lysis buffer (200 mM Tris-HCl pH 8.0, 250 mM NaCl, 25 mM EDTA, 1% SDS) for each gram of mycelia. The mixture was incubated for 15 min at 70 °C and then extracted once each with phenol, phenol:chloroform (1:1) and chloroform. The DNA was precipitated with 100% ethanol and pelleted by centrifugation at 12,000 g for 20 min. The recovered DNA was washed with 70% ethanol and, after drying, dissolved in TE buffer (10 mM Tris-HCl, pH 7.6; 1 mM EDTA, pH 8.0). RNA contamination was eliminated by incubating the samples with RNAse (50 µg/mL) at 37 °C for one hour.

RAPD reactions

After optimizing amplification conditions, the reagent mixture was made up to 25 µL with autoclaved Milli-Q water containing 50 ng DNA, 2.5 µL 10x buffer (Pharmacia), 4 µL dNTP (0.2 mM for each), 2 µL primer (0.4 µM), 0.4 µL Taq polymerase (2.0 units, CENBIOT/RS, Brazil) and 5 µL 7.5 mM $MgCl_2$ (to bring the final magnesium concentration to 3 mM) and overlaid with mineral oil. After initial denaturation at 92C for 4 min, DNA amplifications were performed for 40 cycles of one minute at 92 °C, one minute and 30 s at 37 °C and two minutes at 72 °C. The cycles were followed by a final extension of 3 min at 72 °C. The primers (Operon Technologies, Alameda, CA) used were OPX6 (ACGCCAGAGG), OPX8 (CAGGGGTGGA), OPX11 (GGAGCCTCAG), OPX12 (TCGCCAGCCA), OPX14 (ACAGGTGCTG), OPX17 (GACACGGACC) and OPX19 (TGGCAAGGCA).

Jaccard similarity coefficient (Jaccard, 1908) and the unweighted pair-group method with averages (UPGMA) were used to analyze genetic variability and a dendrogram constructed using the NTSYS-PC program (Rohlf, 1988) with the Winboot program (Yap and Nelson, 1996) being used for bootstrap analysis.

Results

Isolation

We obtained 407 fungal isolates from a total of 433 leaf fragments, the genera most frequently observed being *Guignardia* (*Phyllosticta*) (27%) and *Colletotrichum* (60%). The remaining 13% of isolates was made up of representatives of several other genera, and mainly consisted of sterile mycelia which meant that they were not identified in this study. The overall leaf infection frequency calculated from the number of infected leaf fragments divided by the number of leaf fragments transferred to culture medium (349/433) was 81%. There was no fungal growth on the seeds placed on culture media, indicating that the endophytes probably did not colonize (and thus were not transmitted by) mandarin or tangor seeds.

Identification and cytology

Twelve of the *Guignardia* isolates were identified as being *Guignardia citricarpa* Kiely (anamorph: *Phyllosticta citricarpa* Van der aa), known to be the causal agent of Black Spot disease in citrus. These isolates were then characterized by their reproductive structures and submitted to molecular analysis using RAPD. The structures observed and their average size in µm were: pycnidia (124.03 x 198.51), pycnidiospore (5.93 x 10.83), appendage (7.90), pseudothecium (244.90), ascus (9.70 x 58.20), ascospore (5.77 x 13.00), spermogonium (97.97) and spermatium (1.55 x 6.79). These measurements agree with the data reported in the literature for *G. citricarpa* (Van der aa, 1973; von Arx, 1974; Punithalingam, 1974; Sivanesan, 1984).

Morphologic details of some of the fungal structures are shown in Figure 1 (a-j). The *G. citricarpa* isolates had multinucleated hyphae and uni, bi, tri and multinucleated pycnidiospore (Figure 1, f-j) and binucleated sexual spores

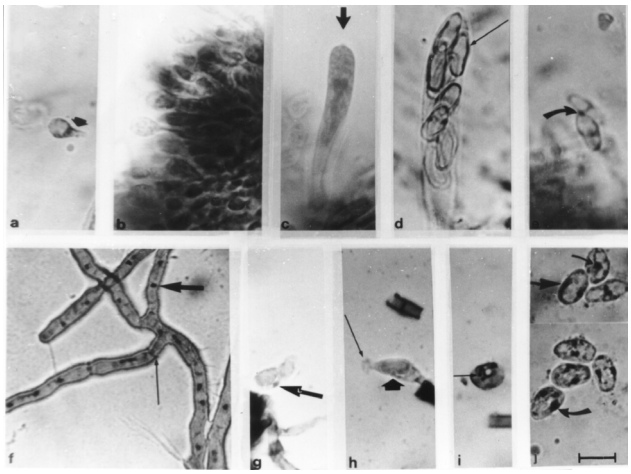

Figure 1 - *Guignardia citricarpa*. Lactophenol staining (a-e.): (a) pycnidiospores, the characteristic appendix (arrow) of the genus *Phyllosticta*; (b) conidiophore; (c) young ascus, mucilaginous cap (arrow); (d) mature ascus, 8 ascospores (arrow); (e) ascospore, the mucilaginous cap (arrow) of the two spore ends is a characteristic of the genus *Guignardia*. Giemsa staining (f-j): (f) septa (thin arrow) and multinucleated hyphae (thick arrow); (g) uninucleated ascospores (arrow); (h) binucleated ascospore, one nuclei (thick arrow) cap (thin arrow); (i) pycnidiospore (multinucleated); (j.) pycnidiospores, uninucleated (thin arrow) and binucleated (thick arrows). Bar = 10 µm.

(Figure 1h). The spermatial form, although rare, was also observed. The `X-spores' were found in a halter form, slightly curved and bigutulated at the ends (Kiely, 1948 and Van der aa, 1973). The isolates showed differences in growth rates (data not shown) needing approximately four weeks to develop mature pseudothecium. However, isolate M86 showed higher production of mature asci after only a week of growth at 28 °C in the dark on PDA enriched with yeast extract. The presence or absence of sexual reproduction was also variable, asci being present in nine *G. citricarpa* isolates but absent in three.

RAPD

The seven primers generated 156 bands (Figure 2) of which 143 were polymorphic, giving a monomorphism rate of about 8%. These data were used to construct a similarity matrix and a dendrogram (Figure 3). Bootstrap analysis indicated that the 12 isolates were grouped into two well-defined clusters with a P value superior to 95%, where P is the percentage appearance of a cluster in 2000 dendrograms generated by bootstrap sampling.

Discussion

After Giemsa staining we found variability in the number of nuclei in asexual and sexual spores of *G. citricarpa*, with approximately half of them becoming binucleated or trinucleated after one or two divisions of their original nucleus (Figure 1f-j). However, for the type-species for this genera, Janex-Favre *et al.* (1993) found that young pycnidiospores (*Phyllosticta bidwelli*) are uninucleated.

By visual inspection some of our isolates, mainly isolate M86, showed a higher growth rate and earlier sporulation compared to others ; this accentuated growth and maturity rate differs from that reported in the literature for pathogenic *Guignardia* species. The citrus pathogen most frequently described in the literature is *G. citricarpa* (Frean, 1966; Kellerman and Kotzé, 1977; Tsai, 1981;

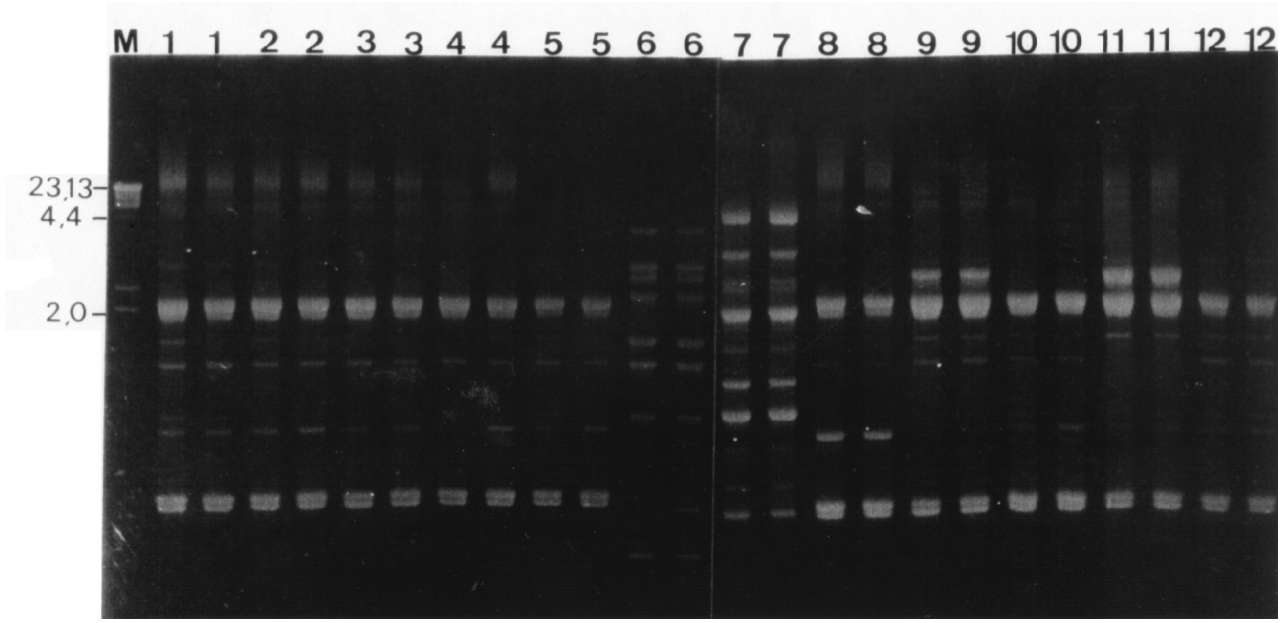

Figure 2 - Polymorphism obtained by amplification of *Guignardia citricarpa* DNA using primers OPX11 (a) and OPX12 (b). Isolates: 1 = P15, 2 = P45, 3 = X68, 4 = X80, 5 = M86, 6 = M131, 7 = C145, 8 = X171, 9 = P210, 10 = M216, 11 = C224, 12 = C320. M = size marker, Hind III/ Eco R1 digested Lambda DNA. B = control reaction without DNA template.

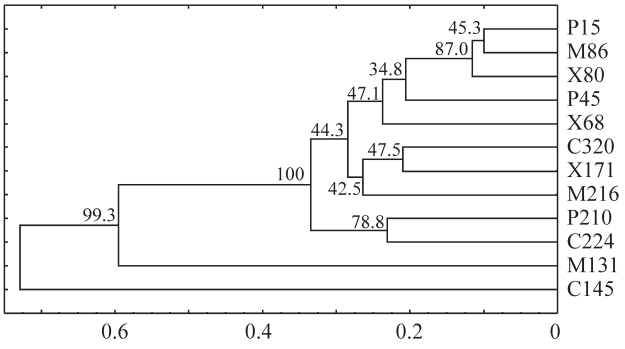

Figure 3 - Genetic distance dendrogram showing the relationships between *Guignardia citricarpa* isolates P15, P45, X68, X80, M86, M131, C145, X171, P210, M216, C224 and C320. The numbers to the left of the clusters are P values and the abscissa Jaccard's similarity coefficient.

Robbs and Bittencourt, 1995), but isolates of this fungus have been described in countries where symptoms of citrus black spot have not been observed and also from asymptomatically infected leaves from twenty plant families. When we collected our isolates only lemon trees (the most susceptible and first to show the disease in orchards) showed symptoms of this disease, no mandarin trees being infected.

McOnie (1964) reported the latent occurrence in citrus (and 13 other hosts) of *Guignardia* isolates (which he called *Guignardia* sp.) easily confused with *G. citricarpa,* although McOnie's isolates did not produce symptoms of black spot disease. When McOnie compared the non-pathogenic isolates with pathogenic *G. citricarpa* (*i.e*. those producing black spot lesions) it was seen that in spite of their being morphologically identical they were physiologically and pathogenically different, McOnie's *Guignardia* sp. isolates producing asymptomatic infections, while the pathogenic isolates caused both symptomatic and asymptomatic infection. Furthermore, according to McOnie the phytopathogenic isolates seemed to infect only citrus while the non-pathogenic had a wide range of hosts.

It thus seems that there are two alternatives regarding our *G. citricarpa* isolates, firstly our isolates may be phytopathogenic but have remained latent in some mandarin plants or, that the isolates are non-phytopathogenic like those described by McOnie (1964). Recent results have shown that the second alternative seems to be the most appropriated one, being nonpathogenic isolates of *G. citricarpa* identified as *G. mangiferae* (Baayen *et al.*, 2002)

The dendrogram generated by RAPD analyses (Figure 3) shows high intraspecific variability. According to bootstrap analysis, the majority of the isolates are grouped in a very definite cluster the internal structure of which could not be defined, although isolates M131 and C145 are distinguishable from each other as well as from the other isolates. Comparing Figure 3 with the citrus varieties from which the isolates were obtained showed no apparent association between the 12 isolates and the varieties from which they were isolated, e.g. isolates P15 and M86 had a high similarity coefficient of 90% but were isolated from different varieties while isolates M86 and M131 had a low with similarity coefficient of 38.8% but came from the same host.

We found no relationship linking morphology with the host plant or genetic similarities of the 12 isolates, which is similar to that which was found for *Hirsutella rhossiliensis* by Tedford *et al.* (1994) who found no link between morphology and pathogenicity. According to Petrini (1991), in some cases the high electrophoretic variability within one *Phyllosticta* species is apparently not reflected by corresponding morphological differentiation. Although Leuchtmann *et al.* (1992) have shown that, for *Phyllosticta* isolates, culture characteristics are similar in lines of different species these authors have also shown that the distribution of enzymatic phenotype in *Phyllosticta* species is related to the host, where each one of five *Phyllosticta pseudotsugae* hosts were infected by lines with distinct phenotypes not found in isolates of any other host species.

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