Fungal Endophytes in Sweet Orange *Citrus sinensis* (L.) Osbeck in Jujuy-Argentina

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ABSTRACT---- ***According to the interaction established with the plant tissues, the endophytes can be mutualistic or pathogenic fungi. The objectives were to identify the fungal endophytes of the sweet orange leaves [Citrus sinensis (L.) Osbeck] from Jujuy-Argentina, and study the in vitro interactions between the isolates. The asymptomatic leaves were taken from the oldest shoots. Leaf pieces were aseptically plated on agar carrot. Isolation, culture and morphometric measurements were made. For some species, identification was performed by PCR-ITS. Inoculations on leaves and fruit orange were carried out, and antagonism test were conducted between non-pathogenic endophytes and the pathogen G. citricarpa.The fungal endophytes isolated from citrus leaves were: Colletotrichum gloeosporioides, Glomerella cingulata, Lasiodiplodia theobromae, Phomopsis citri, Guignardia citricarpa, Phyllosticta capitalensis, Cladosporium herbarum, Xylaria hypoxylon, X. multiplex, Xylaria sp., Fusarium sp., Penicillium sp., Alternaria sp., Diplococium sp.,and Stenella sp. Xylaria spp., C. gloeosporioides and P. capitalensis were found antagonists against G. citricarpa.***

Keywords--- Endophytes, orange, *Xylaria, Phyllosticta, Guignardia*

1. INTRODUCTION

The plant-endophyte interaction is characterized by its asymptomatic nature, its mechanism of action is not clear, but it has been recognised to have a high adaptive potential for host plants against adverse abiotic and biotic factors [22]. The composition of endophytic communities varies depending on the host and the environment. The host and endophytes interact in a harmonious balance, and the loss of that balance can affect the behaviour of the endophyte community members. This can generate conditions for opportunistic fungi to show their pathogenic potential [19]. Experiments have shown that host plants inoculated with endophytes are often more resistant to pathogen and herbivore damage [16]. Endophytic fungi also benefit plants under drought or nutrient stress [5].

Taxonomically most endophytes are Ascomycetes, and some genera are isolated as fungal endophytes always beyond the host [9].

Some fungal endophyte species are frequently reported as pathogens on the same or different hosts, and thus may be pathogens in a latent phase of their life cycle. Therefore, characterizing fungal endophytic communities and their interactions is crucial to understanding fungal diseases of the host plant and is a prerequisite for best management practice [5].

As the endophyte diversity depends on geographic situation and the host identity, the objective was to identify the foliar fungal endophytes from sweet orange *Citrus sinensis* (L.) Osbeck in citriculture area of Jujuy-Argentina, and study their interactions *in vitro*. In this work, *C. gloeosporioides* was found as foliar endophyte dominant specie, and antagonism of the endophytes with *G. citricarpa* was observed.

2. MATERIALS AND METHODS

2.1. ***Isolation and culture of fungal endophytes.***

Sweet orange leaves were collected taken during growing season from 22 study sites of the following localities: Palmasola (24°00'73"-64°20'29"); Palmasola (23°55'38"- 64°16'19"); Cerro la lumbre (24°00'33" - 64°20'52"); Puesto

nuevo (23°54'48"- 64°17'27"); Palmasola (23°58'90"- 64°19'18"); Real de los toros (24°58'04" - 64°19'22"); Sauce guacho (24°16'62" - 64°38'58"); Real de los toros (24°00'72" - 64°20'29"); Real de los toros (24°00'81" - 64°20'27"); Real de los toros (24°00'32" - 64°19'84"); Real de los toros (24°00'09" - 64°20'39"); Yuto (23º 38' 39" - 64º 27' 54"); Yuchán (23º 56' 15" -64º 51' 47"); Fraile Pintado (23°59'09"- 64°47'27"); Chalicán (23º 55' 26" - 64º 50' 37"); El pongo (24º 20' 15" - 65º05'22).

The area is characterized by a warm climate with an annual mean temperature amongst 18.7 and 20.3 ° C and total annual mean precipitation between 600 and 800 mm. In each site, 50 asymptomatic leaves were sampled from the oldest branch shoots of 5 trees. The samples were stored at 4° C until processing in the laboratory. The leaves were washed thoroughly with tap water and neutral detergent, disinfected with sodium hypochlorite 1.5% for two minutes, and then the leaves were washed with 70% ethanol for two minutes and rinsed with sterile distilled water. The leaves were aseptically cut into 1 cm2 pieces that were again disinfected with alcohol and rinsed with distilled water. Then 25 leaf fragments were put on each of 5 carrot agar plates (dextrose 80 g, carrot 200 g, distilled water 1 litre) [21]. To confirm whether the sterilization process was succeful, the final rince water was plated onto carrot agar.

Plates were incubated at 27 ± 1° C for 4 weeks and were ckeked daily for hyphal growth. Emerging hyphae were transferred onto PDA (dextrose 18 g, potato 200 g, distilled water 1 litre) to study cultural characteristic and microscopy. The data were used to group isolated into morphotaxa, wich were identified from morphology when diagnostic characters were evident [6,22].

Differentiation of *Guignardia* species was accomplished by the rate of growth on PDA after 14 days, using three replicates per strain. The average orthogonal diameter of each colony was calculated. In addition, the yellow halo on OA (rolled oats 30 g, agar 15 g, and distilled water 1 L) was observed, and molecular characterization was made [3, 8, 12, 17].

2.2 ***Molecular characterization.***

Fourteen days colonies were suspended in sterile distilled water and 500 µL was plated on agar-water medium (15 g agar, and distilled water 1 litre). After 24 h at 27 ± 2 ° C, germinated conidia were transferred to PDA plates and incubated for 7-15 days at the same temperature. DNA was extracted from conidial cultures to perform polymerase chain reaction (PCR).

The ITS sequences of *Guignardia citricarpa* rDNA were amplifying with a primer 1 (GCF3) [5'-AAA AAG CCC CCG CTA GAC CCT-3'] and a primer 2 (GCR7) [5'-TGT CCG GCG GCC AG-3'] provided by Eurofins. The ITS sequences of *Phyllosticta capitalensis* rDNA were amplifying with a primer 1 (GCF2) [5'-TAA CTT TTG CTA GGT AAA TCC AGA GT-3'] and a primer 2 (GCR4) [5'-CCT GGA TCA CAC AAA ATG AAT TCT T-3']. The reactions were performed in a final reaction volume of 25 µL containing: bidistilled water 12.2 µL; 10 × PCR buffer 2.5 µL; 25 mM MgCl2 1.5 µL; 600 mM dNTPs 2.5 µL; 60 mM primer 0.25 µL; *Taq* DNA polymerase (Promega Corporation) 0.8 U; and genomic DNA 5 ng.

The thermocycler (Bioer Co Mod TC-**/H) conditions used for *G. citricarpa*, were: 120 s at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 59°C, 60 s at 72°C, and a final elongation for 60 s at 72°C. The working conditions for *P. capitalensis* were: 120 s at 94 ° C, followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, 60 s at 72°C, and then 60 s at 72 ° C.

The PCR products were examined by electrophoresis in agarose gel, where a band of 490 base pairs (bp) corresponded to *G. citricarpa*, and a band of 210 bp corresponded to *P. capitalensis* [3, 11,12, 17] .

2.3. ***Evaluation of pathogenicity.***

For suspected pathogens, micro-injuries on detached fruits and seedling orange leaves were done and then inoculated with fragments of 6 mm colonies grown for 7 days in PDA. Then were incubated under appropriate conditions for manifest the disease symptoms.

All 14 days *Guignardia* cultures on PDA plates were transferred to AO for testing pathogenicity and incubated in the dark at 24 ± 2 ° C. After 2 weeks the plates were examined for the presence of a yellow halo on the edge of the colonies [2, 8].

2.4. ***In vitro antagonism.***

Dual culture tests were conducted on PDA plates to verify whether antagonist activity exist between the pathogenic *G. citricarpa* and endophytes, to evaluate antagonism two mechanisms: 1) location on the scale of Bell *et al* (1982) and B) antibiosis. To test the antibiotic effect the percentage of growth inhibition (PIC) at seven days was evaluated, it was calculated by the formula where PIC = [(C1-C2) / C1] × 100, where C1 is the radius of the control colony and C2 radial growth of the pathogen in dual culture treatments [13]. Plates were incubated at 27 ± 2 °C and 7 days. The design was

completely random with four replicates per treatment (each antagonists isolates) and absolute control of the pathogen. The experimental unit consisted of a Petri dish. The results of PIC were analyzed by analysis f variance and comparison of means Tukey to determine significant differences between treatments. Prior to the analysis of variance the data were Log10 undergoing transformation to its standardization, Infostat statistical package was used.

3. RESULTS AND DISCUSSION

3.1.Isolation and culture of fungal endophytes.

The following endophytes were isolated from sweet orange leaves: *C. gloeosporioides* and its teleomorph *Glomerella cingulata* (Stonem.) Spaulding & v. Schrenk; *P. capitalensis* Henn.; *G. citricarpa* Kiely; *Lasiodiplodia theobromae* (Pat.) Griffon & Maudi; *Phomopsis citri* H.S. Fawe. nom. cons.; *Xylaria hypoxylon* (L.) Grev., *Xylaria multiplex* (Kunze) Fr; *Cladosporium herbarum* (Pers.) Link; and species of genera *Alternaria* Nees, *Diplococcium* Grove, *Fusarium* Link, *Penicillium* Link, *Stenella* Syd. and *Xylaria* Hill ex Schrank.

C. gloeosporioides s. lat. was found as foliar endophyte dominant specie ($p \geq 0{,}001$) recovered from asymptomatic leaf tissues at all sites and was in 95% of the fragments analysed. It is the causal agent of anthracnose on citrus fruits and others [24], has been shown to endophyte in mango, plam, coconut fruits [18]. The pathogen causing antracnosis is involved with around the world has high morphological variability and instability in biochemical tests also has the ability to cause latent infections [20]. In *Theobroma cacao* L., a dominant foliar endophyte of this genus was also found, *C. tropicale*, wich increase host defense against pathogens and herbivores attack [16].

(Mejía *et al* 2008) [15] and Wright *et al* (1998) [26] suggested that the composition of endophytes present in a host may be affected by the practices used to control diseases, but this study was conducted in plantations of small growers who do not use fungicidal treatments.

L. theobromae and *P. citri* were recovered as an endophyte in 4.7% of sites with a frequency below 1% of the fragments examined, both fungi cause rotting of citrus fruit and are often isolated from asymptomatic tissue.

The *Xylaria* species were obtained from asymptomatic leaves in 13% of sample sites with a frequency of 4%. *X. hypoxylon* and *X. multiplex* formed fruiting bodies on leaf pieces and CA. Another was not identified at the species level. The *Xylariaceae* seem to exist only as endophytes with no obvious benefit to the host. *Xylaria* can act as a latent coloniser that causes the breakdown of tissues in the plant and initiates senescence. Some endophytic strains of *Xylaria* produce terpenes and metabolites of diverse chemical nature, with antimicrobial activity against various phytopathogenic fungi [14,27].

Two species of *Phyllosticta*, *P. citricarpa* (=*G. citricarpa*) and *P. capitalensis*, were isolated and developed anamorphic colonies on PDA. *G. citricarpa* was obtained from asymptomatic orange leaves in 4.7% of the sites, with a frequency that did not reach 1%. Its anamorph developed black, stromal, slow-growing colonies, with a lobed margin and picnidia with cirrus and the average growth rate was 0.2 cm/day. The elliptical conidia were hyaline, guttulate, pedicellate, with 9-13 x 5-8 µm in size, surrounded by a colourless layer were released. It is a pathogen of both orange and other citrus plants and it has been isolated from latent infections [2, 3, 8]. *P. capitalensis* colonies were black or olive green, with diffuse edges. The conidia were elliptical, guttulate of 8-11 x 6-9 µ surrounded by a mucilaginous cover. The average growth rate of colonies was 0.4 cm/day. It was recovered as endophyte in 13% of sites sampled, with a frequency of 30%.

P. capitalensis and *G. mangiferae* were recovered as endophytes from several tropical and subtropical plants, including citrus plants [23], although some authors describe both as pathogenic to lemon, causing mottled and reddish spot [3, 7]. Alcoba *et al* (2000) [1] considered the mottled and reddish spot as symptomatic variations of black spot caused by *G. citricarpa.* Phylogenetic analyses of various endophytic and pathogenic isolates indicate that *P. capitalensis* and *G. mangiferae* are distinct species [9].

C. herbarum was isolated from 4.7% of sites sampled. It was described as an endophyte in citrus and other plants [21]. *Penicillium* sp., *Fusarium* sp. and *Cladosporium* sp. were recovered erratically at very low prevalence and frequency.

3.2 ***Molecular characterization.***

Phyllosticta isolates were differentiated by their morphological and cultural characteristics into two groups: *P. capitalensis* and *P. citricarpa.* This was confirmed by the sequence homology of the ITS1-ITS2 conventional PCR with specific bands on agarose gels [17]. Both species were found simultaneously on the same host but not with the same symptom, as indicated [3].

G. citricarpa strains characterized by homology PCR ITS1, ITS2, formed an yellow halo on OA, but not those identified as *P. capitalensis (ex G. mangiferae),* in agreement with the findings of others authors [25, 28].

3.3. ***Evaluation of pathogenicity.***

The fruits inoculated with *L. theobromae* showed the typical symptoms of stem end rot disease. Mummified fruits developed the typical black pycnidia of the species. *P. citri* caused a firm rot with pycnidia exuded cirrus conidia α and β typical of this species. *C. gloeosporioides* produced postharvest fruit rot, no symptoms in leaves but it could be reisolated from them.

The *Xylaria* inoculated leaves and fruits not develop disease symptoms, but the fungus could be recovered from leaves at the end of the experiment causing a decomposition of the tissues. These results are in accordance with Liers *et al* (2006) [14] who consider them asymptomatic colonizers.

The assessment of pathogenicity confirmed that *P. capitalensis* is an endophyte in sweet orange, but Fogliata *et al* (2004; 2006) [9, 10] believe it is a lemon pathogen.

3.4. Invitro evaluation of antagonistic capacity.

The category substrate competition according to the class of Bell et al. (1982) [4] and percent inhibition of pathogen growth (PIC) of *P. capitalensis*, *C. gloeoporioides* and *X. multiplex* against *G. citricarpa*, , is done in Table 1. The antagonism is significantly higher with the pathogenic *Phyllosticta* that the endophytic ($p \leq 0.001$).

Table 1. Category substrate competition and percent of pathogen growth inhibition (PIC) of the leaf isolated endophytes sweet orange scale according to the class of Bell *et al* (1982). Treatments with different letter in the PIC have statistical differences at 5%.

Dual cultura	Bel scale	PIC
X. hypoxylon - G. citricatrpa	*Class I*	31,8% b
X. multiplex - G. citricatrpa	*Class I*	42% b
Xylaria sp. - G. citricatrpa	*Class II*	30% c
P. capitalensis - G. citricatrpa	*Class II*	57% a
C. gloeosporioides - G.citricarpa	*Class I*	52,84 a
C. gloeosporioides - P. capitalensis	*Class III*	9 d

These results show that two endophytes coexist better than an endophyte against a pathogen. Competition for nutrients and substrate on the surface of plants, is a mechanism very successful for biological control to ectoparasites, but the effect of antibiosis indicated by the PIC is perhaps more conclusive for foliar pathogens that colonize the host internally as *G. citricarpa* in citrus. However, substrate competition is a strategy that has potential in stage survival of the pathogen, when the abundance of the primary inoculum is a determining factor.

It is difficult to define the type of interaction of a fungus on a host and depends on the diversity of microorganisms that colonize the environment, so it is likely that *P.capitalensis* was found by some authors as endophyte and by others as pathogen in a same host, moreover plants normally harbor many endophyte species [2, 16].

4. CONCLUSIONS

Many fungal species coexist into the *C. sinensis* tissues as endophytes, *C. gloeosporioides* s. lat. was found as the dominant leaf sweet orange endophyte in the study area, it was observed antagonism in front of *G. citricarpa*, but not with *P. capitalensis*. *L. theobromae*, *P. citri* and *G. citricarpa* were isolated on their latent period of the infection cycle since they showed their potential pathogen.

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