Proceedings VI World Avocado Congress (Actas VI Congreso Mundial del Aguacate) 2007.
Viña Del Mar, Chile. 12 – 16 Nov. 2007. ISBN No 978-956-17-0413-8.

GREEN FLUORESCENT PROTEIN APPLIED TO THE STUDY OF COLONIZATION AND INFECTION OF AVOCADO TREE ROOTS BY *Rosellinia necatrix*

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Rosellinia necatrix Prill. is a soilborne ascomycete fungus that causes white root rot on a wide range of plant species, especially on fruit trees. White root rot is currently one of the most important diseases affecting avocado orchards in Andalusia (Spain) as well as apple, grape and pear orchards in Japan. Characteristic symptoms of this disease are rotting of roots, yellowing and falling of leaves, wilting and, finally, death of the tree. Spanish and Japanese isolates of *R. necatrix* showing different degrees of virulence were tagged with the green fluorescent protein (GFP) by protoplast transformation with plasmid pCPXHY1eGFP. Frequencies of protoplasts regeneration and transformation varied greatly among isolates and were $10^{-5}/10^{-7}$ and $10^{-2}/>10^{-3}$ per 10µg of DNA, respectively. Microscopic analysis of the transformants revealed homogeneity of the fluorescent signal, which was clearly visible and stable in the hyphae. Currently, the pathogenicity of wild-type isolates and transformants is analysed, evaluating the disease index after its inoculation in avocado plants. Colonization, infection, and disease development on avocado roots infected with the transformants is being analyzed *in vivo* by scanning confocal laser microscopy; details of these processes will be essential for disease control. To the best of our knowledge, this is the first report describing the utilization of GFP-tagged *R. necatrix* derivatives to analyze the infection process of avocado roots by this pathogen.

Key Words: *Persea Americana Mill*, *Rosellinia necatrix*, White root rot, Green Fluorescent Protein (GFP).

PROTEÍNA FLUORESCENTE VERDE APLICADA AL ESTUDIO DE LA COLONIZACIÓN E INFECCIÓN DE RAÍCES DE AGUACATE POR *Rosellinia necatrix*.

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Rosellinia necatrix Prill. es un ascomiceto de suelo que causa la podredumbre blanca en un amplio rango de especies vegetales, especialmente árboles frutales. La podredumbre blanca es actualmente una de las enfermedades más importantes que afectan a las plantaciones de aguacate en Andalucía (España), así como a las de manzano, vid y peral en Japón. Los síntomas característicos de esta enfermedad son la podredumbre radicular, amarillamiento, decaimiento, caída de hojas, y finalmente, muerte del árbol. Aislados españoles y japoneses de *R. necatrix*, que mostraban diferentes grados de virulencia, se marcaron con la proteína verde fluorescente (GFP) mediante transformación de protoplastos con el plásmido pCPXHY1eGFP. Las frecuencias de regeneración de protoplastos y transformación variaron fluctuando entre $10^{-5}/10^{-7}$ y $10^{-2}/>10^{-3}$ por 10µg de DNA, respectivamente. El análisis microscópico de los transformantes reveló homogeneidad de la señal fluorescente, siendo claramente visible y estable en las hifas. Actualmente, se está analizando la patogenicidad de los diferentes aislados silvestres y transformantes, evaluando el índice de enfermedad tras su inoculación en plantas de aguacate. Paralelamente, se está estudiando la colonización de raíces de aguacate por *R. necatrix*, así como la infección y desarrollo de la enfermedad *in vivo* mediante Microscopía Láser Confocal; los detalles de estos procesos serán esenciales para el control de la enfermedad. Éste es el primer trabajo en el que se describe la utilización de aislados de *R. necatrix* marcados con la proteína fluorescente verde para analizar el proceso de infección en raíces de aguacate por este patógeno.

Palabras Clave: *Persea Americana Mill*, *Rosellinia necatrix*, Podredumbre blanca, Proteína fluorescente verde.

INTRODUCTION

The most important diseases affecting avocado (*Persea americana* Mill) orchards are *Phytophthora* root rot, caused by the oomycete *Phytophthora cinnamomi,* and white root rot, caused by the fungus *Rosellinia necatrix*. While *P. cinnamomi* affects avocado orchards worldwide, infections caused by *R. necatrix* causes considerable damage of avocado trees in the Mediterranean area, mainly in Israel and Spain. Besides, *R. necatrix* has a very wide host-range and is very destructive to many fruit tree crops, including tropical and subtropical species such as avocado and mango. Affected trees show rotting of roots, yellowing and falling of leaves, wilting, and finally, death of the tree within a few weeks after the appearance of the first foliar symptoms.

Currently, available information about the infection process of fruit trees by *R. necatrix* is scarce. Pathologico-anatomical observations of white root rot infection of

mulberry trees has been performed many years ago (Sakurai, 1952). In this study, it was observed that fungal invasion of young roots, which have not developed secondary tissues, occurs by boring and dissolving cork cell walls and, on rare occasions, by wedging them. However, invasion of adult roots into the inner tissues seems to take place mainly through the suberized closing layers of the lenticels, generally as hyphal strands. Damaged cells show brownish discoloration and, finally, necrosis (Sakurai, 1952). Nevertheless, microscopic visualization of the infection process of avocado roots by *R. necatrix* has not been performed to date (Perez-Jimenez, 2004).

The green fluorescent protein (GFP) from the jellyfish *Aequorea Victoria* is a useful tool to tag pathogens in order to visualize, by fluorescence microscopy techniques, their infection behaviour under in vivo conditions. In this respect, visualization of GFP-labelled organisms by confocal laser scanning microscopy (CLSM) is an effective, fast, and non-invasive tool allowing spatiotemporal analysis of pathogen-host interactions.

In this work, we describe the generation of protoplasts and transformation with different GFP-tagged plasmids of several *R. necatrix* strains showing different degrees of virulence. Epifluorescence microscopy of the transformants revealed homogeneity of the GFP fluorescent signal, which was clearly visible in the hyphae. The infection process of avocado roots by a *R. necatrix* was studied using CLSM. This is the first report describing the utilization of GFP-tagged *R. necatrix* derivatives to analyze the infection process of a fruit tree by this pathogen.

Material and Methods

Microorganism and culture conditions

Fungal strains and plasmids used in this study are listed in Table 1. Fungal strains were grown at 25 ºC on potato dextrose agar (PDA; Difco Laboratories, Detroit). Culture media was supplemented with 50 µg/ml of hygromycin B (HYG) when required.

Transformation of Rosellina necatrix

Protoplast generation was carried out as previously described by Kanematsu and co-workers (2004). The conditions used for protoplast preparation were: mycelial cultures were incubated without shaking, mannitol osmotic solution 0.6 M, enzyme-osmoticum mixture contained zymolyase 100T 0.4 % and lysing enzymes 1.5 %. For transformation, protoplast suspension and 10 µg of the corresponding plasmid vector were dispensed in a Falcon tube, mixed gently, and placed on ice for 30 min. Following the addition of 500 µl of PEG solution [PEG 4000], protoplast suspension was gently mixed and incubated at 20 ºC for 20 min. Each tube received 700 µl of regeneration broth (potato dextrose broth, PDB; Difco Laboratories, amended with 0.5 M glucose) and was incubated at 25 ºC for 7 days. Aliquots containing 300 µl of protoplast were plated to 10 ml of YCDA (0,1 % yeast extract, 0,1 % casein hydrolysate, 0.5 M gluocose and 1,5% agar in a 9-cm Petri dish) at 20 ºC in the dark for 2 weeks. HYG resistance (HYG^R) was tested by overlaying PDA regeneration plates with 10ml of PDA medium supplemented with HYG 50.

Microscopic analysis

Microscopic analysis of the hyphae was carried out using a Leica epifluorescence microscope and a stereoscopic fluorescence microscope (Leica MZ FLIII), both equipped with filter blocks with spectral properties matching those of GFP (470/20-nm excitation, 515 Long Pass emission).

Pathogenicity test

Pathogenicity of GFP-tagged *R. necatrix* isolates in comparison with their corresponding wild-type strains was performed in avocado plants using one-year-old, six-month-old and three-month-old avocado seedlings. Seedlings were placed into pots containing substrate infected with *R. necatrix* using wheat grains, as previously described (Freeman et al., 1986, Pliego et al., 2007).

Results and Discussion

Several Japanese and Spanish *R. necatrix* isolates showing different degrees of virulence (Table 1) were used for GFP-labelling. While Spanish avocado isolates CH1, CH2, CH3 and CH4 are highly virulent; CH5 and CH6 do not induce root rot symptoms on avocado plants. RT1 and RT2, which were isolated from diseased roots in Japanese pear orchards, are highly virulent in both pear and avocado trees (data non shown).
Different approaches were carried out for transformation of *R. necatrix* isolates. In a first approach, *Agrobacterium tumefaciens*-mediated transformation of *R. necatrix* mycelia was carried out as described by Zhang et al (2003) for *Glarea lozoyensis* with some modifications. A derivative of plasmid pPK2 containing a Hyg^R gene fused to *gfp* gene was used for transformation of *A. tumefaciens* strain LBA 4404 (Hoekema et al. 1983). After several assays, no fungal transformants were obtained using this method.

In a second trial, protoplast generation and transformation experiments were carried out as previously described (Satoko et al., 2004). About 10^7-10^8 protoplasts per g of wet mycelia were obtained from each of the *R. necatrix* strains assayed (Table 1). Size of the protoplast greatly varied among isolates. While clearly visible and round protoplasts were generated from strains CH2 and CH4, minor protoplasts were produce from isolates CH1 and CH6; protoplast of intermediate sizes were generated for the rest isolates. Protoplast regeneration frequencies varied among the different isolates, reaching values around $10^{-5}/10^{-7}$ for CH1, CH4, CH5, CH6, RT1, RT2 and $10^{-2}/10^{-3}$ for CH2 and CH3. However, a correlation between protoplast size and regeneration frequency was not observed.

Two different GFP-tagged vectors were used for protoplast transformation of *R. necatrix* isolates: a) co-transformation of pGPD-GFP and pAN7-1 plasmids, carrying *gfp* and Hyg^R genes, respectively and, b) transformation with plasmid pCH-GFP, carrying both *gfp* and Hyg^R genes (Table 1). No transformants were isolated for any of the *R. necatrix* strains tested using co-transformation of pGPD-GFP and pAN7-1. However, transformants were isolated for five different strains (CH1, CH5, CH6, RT1 and RT2) using plasmid pCH-GFP. Transformants were neither isolated for CH2, CH3 and CH4 using this plasmid (Table 1).

Table1. Fungal strains and plasmids used in this study

Rosellinia necatrix	**Relevant characteristics**	**Origin**	**Transformants**
CH1	Highly virulent	Spain	CH1-GFP
CH2	Highly virulent	Spain	-
CH3	Highly virulent	Spain	-
CH4	Highly virulent	Spain	-
RT1	Highly virulent	Japan	RT1-GFP
RT2	Highly virulent	Japan	RT2-GFP
CH5	Avirulent	Spain	CH5-GFP
CH6	Avirulent	Spain	CH6-GFP

Plasmids	**Relevant characteristics**	**Reference**	**Transformants**
pGPD-GFP	pAN52-10-S65TGFPn/n derivative containing *gfp* under the control of the *gpdA* promoter.	Lagopodi et al. 2002	Not isolated
pAN7-1	*Escherichia coli* hygromycin-B resistance gene cloned between the *gpdA* promoter and the *trpC* gene from *Aspergillus nidulans.*	Punt et al. 1987	Not isolated
pCHX-GFP	pXH9 derivative containing *gfp* and *E. coli* hygromycin-B resistance gene under the control of the *gpdA* and *trpC* promoters, respectively.	This study	Isolated for five isolates

Epifluorescence microscopy analysis of the transformants isolated revealed homogeneity of the green fluorescent signal, which was clearly visible in the hyphae for all five GFP transformants. Figure 1A shows the results obtained for *R. necatrix* CH1-GFP in comparison with its wild type strain CH1.

GFP expression did not affect pathogenicity of the different transformants, as disease development was identical in avocado plants infected with either the wild type strains or the GFP-transformants. In all avocado seedlings tested (one-year-old, six-month-old and three-month-old), aerial symptoms started by yellowing of leaves, wilting, and finally, death of the plant. Mycelia aggregations invading avocado roots followed by root rot were also observed (Fig. 1B). GFP-CH5 and GFP-CH6 remain avirulent not inducing disease development in any of the cases tested (data not shown).

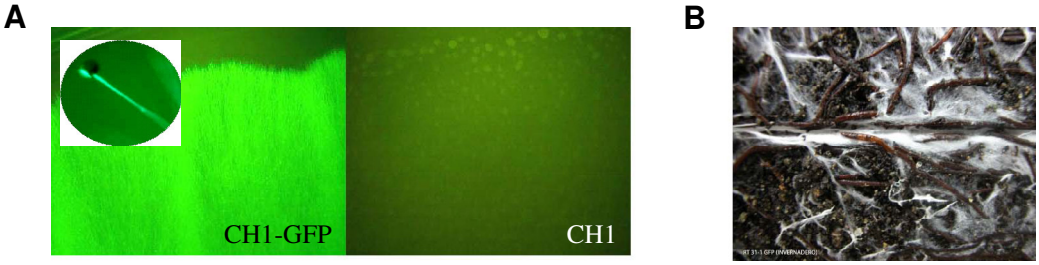

Figure 1. *GFP emission of CH1-GFP and its corresponding parental strain CH1 (A). A detail image of an individual hypha is shown for CH1-GFP. B. White root rot caused by GFP-derivative CH1 in one-year-old avocado seedlings.*

Artificial inoculations of avocado plants with *Rosellinia* GFP-derivatives are under study to visualize the infection process of avocado roots using different epifluorescence microscopy techniques. Preliminary results showed that, as previously described for apple plant roots (Labrouhe, 1982), different phases of disease development could be differentiated on avocado. First of all, external proliferation of the

hiphae along the root surface as a diffuse mycelium or as hyphal strands was observed Fig. 1B). Secondly, penetration of mycelial aggregates on young tissues seems to occur at several points of the root located between epidermal cells. Penetration was followed by an invasion and disorganisation of cortical parenchyma (data not shown). Further investigations approaching *R. necatrix* penetration into older avocado root tissues are in progress. A detail understanding of *R. necatrix* infection of avocado roots will contribute to the development of more efficient ways to control white root rot disease, as it has been demonstrated for biological control of other fungi, i.e. *F. oxysporum f. sp. radicis-lycopersici* on tomato roots (Bolwerk A. et al. 2003).

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