RESEARCH

Molecular diagnosis of Fusarium *spp. isolates associated to bud rot of Oil palm in Ecuador*

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ABSTRACT

Oil palm (*Elaeis guineensis* Jacq), is an important crop worldwide due to high oil production. The disease named bud rot (BR) or complex BR is a morpho-physiological disorder, causing the total destruction of commercial plantations and significant economic losses in countries like: Panama, Colombia, Suriname, Ecuador and Brazil. Complex BR is characterized by an initial yellowing, followed by necrosis and rot of the spear (arrow shape), the disease affecting the bud and causing plant death. The aim of this work was to molecularly characterize *Fusarium* spp. infections associated to the BR in oil palm in San Lorenzo (Esmeralda Province, Ecuador), by using a molecular characterization technique. Fungal isolates were identified by PCR-based assays and DNA sequencing of the ITS region, also being characterized by morphological observations of cultures in PDA medium. Molecular analysis identified *Fusarium proliferatum* (isolate PASL 0712) and *Fusarium* sp. (isolate PASL 0112) associated with oil palm BR in Ecuador.

Keywords: bud rot, *Fusarium proliferatum*, oil palm, ITS

Biotecnología Aplicada 2015;32:2221-3

RESUMEN

Diagnóstico molecular de aislados de *Fusarium* **spp, asociados con la pudrición del cogollo de la Palma aceitera.** La palma aceitera (*Elaeis guineensis* Jacq), es un cultivo de gran importancia agrícola en todo el mundo, pues alcanza una elevada producción de aceite. La enfermedad conocida como pudrición del cogollo (PC) o complejo PC en *E. guineensis*, causa alteraciones morfo-fisiológicas que conllevan a la destrucción total de las plantaciones y provoca pérdidas económicas en países como: Panamá, Colombia, Surinam, Ecuador y Brasil. El complejo PC, se caracteriza por una coloración amarilla de las hojas más jóvenes, seguida de una necrosis y pudrición de la flecha (hoja sin abrir), la enfermedad puede descender hacia el cogollo y provocar la muerte de la planta. Con el objetivo de determinar los agentes biológicos asociados con los síntomas del complejo PC, se realizó la identificación molecular de los aislamientos obtenidos. Estos se caracterizaron mediante la técnica de PCR y secuenciación del ADN de la región ITS, además de las observaciones morfológicas de cultivos en medio PDA. Se identificó a los aislamientos PASL 0712 como *Fusarium proliferatum* y PASL 0112 como *Fusarium oxysporum* species complex, asociados al complejo PC en la palma aceitera en Ecuador.

Palabras clave: pudrición del cogollo, *Fusarium proliferatum*, palma aceitera, ITS

Introduction

Oil palm (*Elaeis guineensis* Jacq) is an economically-relevant monocotyledonous crop due to its high oil production [1], and particularly in Ecuador, where its plantations extend for approximately 240 000 ha [2].

The disease known as bud rot (BR) or complex BR is a morpho-physiological disorder, causing significant economic losses and the total destruction of commercial plantations in Latin America, particularly in Panama, Colombia, Suriname, Ecuador and Brazil [3]. Complex BR is characterized by chlorosis and yellowing of the young fronds surrounding the bud [3, 4], followed by the rotting and gradual desiccation of the spear at advanced disease stages, causing plant death.

Currently, no primary pathogens have been identified as the causative agent of BR-related symptoms [3, 4], in spite of reports on pathogens affecting this species, such as: *Fusarium bulbigenum* var. *tracheiphillum* (E. F. Smiith) Wr, *F. dimerum* Penzig, *F. oxysporum* f.sp. elaeidis Toovey, *Fusarium solani* (Martius) Appel & Wollenweber emend. Snyder & Hansen, and *Fusarium vasinfectum* Atk [5]. In fact, no fungi species have been molecularly identified as causing RC in nursery conditions for the oil palm hybrid Coarí × La Mé.

For this purpose, this work was aimed to identifying microorganisms associated to the symptoms of the RC complex in *E. guineensis*, Coarí × La Mé hybrid) with the aid of molecular characterization procedures.

Materials and methods

Fungi isolation

Samples were taken from 6-months-old plants of *E. guineensis* Coarí × La Mé hybrid plants cultivated under nursery conditions in facilities of the Palmeras de Los Andes enterprise, and affected by the bud rot. For the isolation of fungi present, samples of the bud and the spear were cut, washed with tap water for a few minutes, further disinfected with 2 % sodium hypochlorite for 10 min and washed again with sterile distilled water.

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Subsequently, the samples were dried in sterile filter paper into an air flow cabinet and cut with a sterile scalpel. The sections of the infected vegetal material were seeded in 2 % Agar-Water culture medium (Difco, cat. No. 281230) for mycelia growth of the fungi isolates. Once mycelia were developed, a fragment was transferred to 90-mm Petri dishes filled with Potato Dextrose Agar broth (PDA; Scharlau, cat. 01-483). Monoconidia were isolated to obtain pure monospore cultures, as described by Benson [6]

Culture and morphological characterization of isolated fungi

The pure fungi isolates in culture were characterized, by morphology observation of the colonies obtained, particularly their mycelial growth, color of the culture medium, presence and shape of macro- and microconidia, chlamydospores and phialides, as previously described [7-10].

Molecular identification of fungi associated to the RC complex in the oil palm

For the molecular identification of monosporic cultures of the PASL 0112 and PASL 0712 fungi isolates, 20 × 100 mm Petri dishes (Anumbra) containing 10 mL of PDA medium were seeded from fungi cultures, incubated for 15 days, and a mycelium disc of 0.5 cm in diameter was taken for each isolate.

Afterwards, discs were placed in 250-mL erlenmeyers (Boeko) containing 100 mL of liquid B5 medium supplemented with 2 % sacarose (w/v/), and further incubated at 25 ºC for three weeks under agitation at 120 rpm in an orbital shaker. Then, mycelia were collected by filtration and stored frozen at –20 ºC until use.

Total genomic DNA was extracted with the Trizol DNA extraction kit (Sigma-Aldrich Co., USA) following the manufacturer's instructions (Invitrogen). DNA concentration was determined by measuring the absorbance at 260 nm in a spectrophotometer (Ultrospec Plus Spectrophotometer Pharmacia, LKB) as described [10].

The internally transcribed spacer (ITS) rDNA sequences were amplified by using a PCR reaction mix in a final volume of 25 µL, containing: 10× PCR buffer 10 mM tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$); 200 µM dNTPs; 100 ng DNA; the specific primers ITS4 5´-TCCTCCGCTTATTGATATGC-3´) and ITS5 (5´-GGAAGTAAAAGTCGTAACAAGG-3´) [11], 10 µM each and 1 U of Taq polymerase (Amplicen). Amplification was made in a PTC-100 termocycler (M J Research, Inc.), under the following conditions: initial denaturation at 94 ºC for 30 s; a hybridization step at 55 ºC for 30 s and an extension at 72 ºC for 1 min. A final extension step was run at 72 ºC for 7 min [12]. PCR bands of 587 bp for isolate PASL 0112 and 582 bp for PASL 0712 were obtained (data not shown).

The PCR amplification products were purified with the QIAquick® kit (Qiagen, Germany) following the instructions of the manufacturer, and both strands each were sequenced with primers ITS4 and ITS5 in an ABI Prism sequencer, model 377, version 2.1.1 (Applied Biosystems; Warrington, United Kingdom). The obtained sequences were finally compared by a BLAST analysis against the GenBank® database.

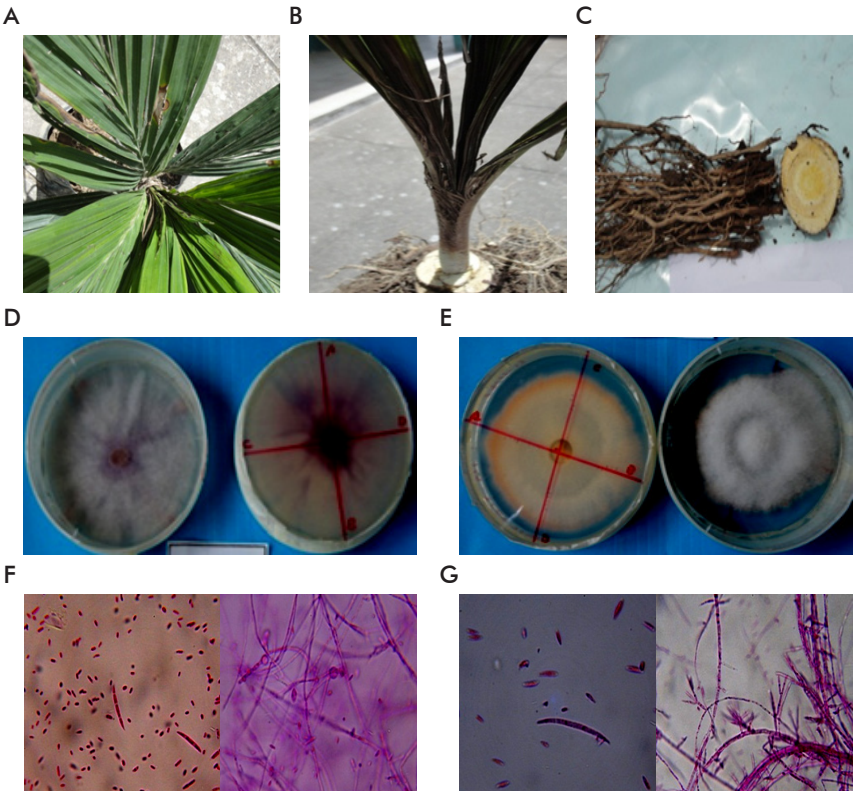

Figure. Bud rot disease in Oil palm (*Elaeis guineensis* Jacq) Coarí × La Mé hybrid plants and characterization in culture medium of the associated *Fusarium* spp. fungi isolates. Isolates were obtained from disease tissue samples and cultured in PDA medium. A) disease manifestation in the spear leaflets and the bud of the plant. C) light yellow root base and meristematic areas of palms affected by the disease, with necrosis in primary, secondary ans tertiary roots. D) and E) Culture characterization of fungi isolates PASL 0112 and PASL 0712 in solid PDA medium cultures, respectively. F) Morphological characterization of isolate PASL 0112 (left image: macro- and microconidia; right: mono- and polyphialides). G) Morphological characterization of isolate PASL 0712 (left image: macro- and microconidia; right image: phialides and chlamydospores).

Results

Culture and morphological characterization of isolated fungi

Plants showed symptoms of chlorosis, with necrotic sections in the leaflets surrounding the spear (Figure 1A). The base of the root and the meristem was light yellow, with necrosis in primary, secondary and tertiary roots (Figure B and C).

In the case of PASL 0112 isolate, a cottony mycelium of pale violet pigmentation was seen in culture (Figure D), presenting long and straight macroconidia (3-septate, 29.81 × 2.55 µm), ovoid and ellipsoidal microconidia (8.56 µm × 2.77 µm), short monophialides. Chlamydospores were abundantly-smooth or verrucose, globose to subglobose, and single (7-11 µm). They were present in hyphae (either terminal or intercalary), in pairs, short chains or occasionally in clumps (Figure F) [8, 9].

The colonies of the PASL 0712 isolate developed abundant cottony mycelia, with pale orange pigmentation in culture (Figure E). The morphological characterization showed the presence of macroconidia long with parallel ventral and dorsal walls and slender (3-septate, 30.78 × 2.55 µm), the apical cell

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12. Leslie JF, Summerell BA. The Fusarium Laboratory Manual. Oxford: Blackwell Publishing; 2006.

was conical, slightly pointed and with a foot-shape at basal cell, 3-7 septate (usually 5). Microconidia were ovoid with flattened base (7.24 µm × 2.55 µm), assembled forming short chains or false heads. Presence of mono- and polyphialides. Chlamydospores were absent (Figure G) [8, 9].

Molecular identification of fungi associated to the RC complex in the oil palm

The DNA sequence of fragments amplified with primers specific for the ITS rDNA region of the genomic DNA of *Fusarium* spp. were successfully matched with GenBank® database sequences. They corresponded to *Fusarium proliferatum* (Matsushima) Nirenberg fungi for the isolate PASL 0712. But in the case of the PASL 0112 isolate, it was corroborated as corresponding to *Fusarium* spp., in spite of the culture-morphology characterization (Table), the match sequence in the database being species unspecific (*Fusarium oxysporum* species complex). Both matches showed a 99 % of homology with sequences deposited under GenBank® accession numbers HF930594.1 (PASL 0712) and EU236709.1 (PASL 0112) (Table).

Discussion

The identification of *Fusarium* spp. species based solely on culture and morphological criteria tends to be confusing, even for well-trained taxonomists, despite their correspondence with previous descriptions [8, 9], as was the case for the PASL 0712 and PASL 0112 isolates.

That's why a genomic DNA characterization must be made to differentiate among the species showing similar morphological parameters, as for *F. proliferatum* and *Fusarium moniliforme* Sheldon emend. Snyder & Hansen. In fact, *F. proliferatum* have been mistakenly identified as *F. moniliforme* several studies due to their high sequence homology, in addition to both species remaining as almost undistinguishable in terms of their biological and morphological parameters [13, 14].

In this sense, the ITS rDNA sequences were used for the molecular identification of the fungi isolates obtained from oil palm bud rot samples [15]. This method it a fast and reliable way to characterize both genus and species, due to the typical distinct variability of ITS between species [15].

Table. Sequences and homology match of the ITS rDNA region of genomic DNA sequencing of *Fusarium* spp. isolates for the molecular characterization of fungi associated to the Oil palm bud rot in Ecuador

Isolate	Primer	Sequence (5´-3´)	GenBank® match*	Species
PASL 0112	ITS5	CCGGGATGGTGACAGCGGAGGGACATTACCC-GAGTTTACACTCCCAAACCCCTGTGAACTACCAATT-GTTGCCTCGGCGGATCAGCCCGCTCCCGGTAAA-ACGGGACGGCCCGCCAGAGGACCCCTAAACTCT-GTTTCTATATGTAACTTCTGAGTAAAACCATAAATAA-ATCAAAACTTTCAACAACGGATCTCTTGGTTCTGG-CATCGATGAAGAACGCAGCAAAATGCGATAAGTAAT-GTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGA-ACGCACATTGCGCCCGCCAGTATTCTGGCGGGCAT-GCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCC-CGGGTTTGGTGTTGGGGATCGGCGAGCCCTTGCGG-CAAGCCGGCCCCGAAATCTAGTGGCGGTCTCGCTG-CAGCTTCCATTGCGTAGTAGTAAAACCCTCGCAACT-GGTACGCGGCGCGGCCAAGCCGTTAAACCCC-CAACTTCTGAATGTTGACCTCGGATCAGGTAGGAA-TACCCGCTGAACTTAACAACGAGGGGGGGGGGG-GAAAAAAAAAAACTGAGGGTTCACCCCCCCCCTCC	EU236709.1	*Fusarium oxysporum* species complex
PASL 0712	ITS4	GGGGACTGGGATTCTACCTCGATCGAGGTCACATT-CAGAAGTTGGGGGTTTAACGGCTTGGCCGCGC-CGCGTACCAGTTGCGAGGGTTTTACTACTACGCAATG-GAAGCTGCAGCGAGACCGCCACTAGATTTCGGGGC-CGGCTTGCCGCAAGGGCTCGCCGATCCCCAACAC-CAAACCCGGGGGCTTGAGGGTTGAAATGACGCTC-GAACAGGCATGCCCGCCAGAATACTGGCGGGCG-CAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCT-GCAATTCACATTACTTATCGCATTTTGCTGCGTTCTT-CATCGATGCCAGAACCAAGAGATCCGTTGTTGAA-AGTTTTGATTTATTTATGGTTTTACTCAGAAGTTACA-TATAGAAACAGAGTTTAGGGGTCCTCTGGCGGGC-CGTCCCGTTTTACCGGGAGCGGGCTGATCCGCC-GAGGCAACAATTGGTATGTTCACAGGGGTTTGG-GAGTTGTAAACTCGGTAATGATCCCTCCGCTGGTT-CACCAACGGAGACCTTGTAATTTTTTTTTTTTTC-TAAAAAACCCCTGGTTAAAATTACCAA	HF930594.1	*Fusarium proliferatum*

* Both sequence matches displayed a 99 % sequence homology by BLAST analysis.

Our results confirmed the spear and bud necrosis disease in the oil palm Coari × La Mé hybrid as associated to the presence of *Fusarium* spp., corroborated with molecular DNA amplification by PCR and sequence alignment of ITS sequences. Both isolates showed 99 % of similarity with previously reported sequences of this genus, in spite of isolate PASL 0112 species remaining to be further established (Table).

As far as we know, this was the first report on the association of this *F. proliferatum* fungi species as part of the fungi complex associated to the oil palm bud rot disease affecting *E. guineensis* in Ecuador.

Received in January, 2015.
Accepted in July, 2015.

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