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Pestalotiopsis theae (*Ascomycota*, *Amphisphaeriaceae*) on seeds of *Diospyros crassiflora* (*Ebenaceae*)

Clovis Douanla-Meli & Ewald Langer

douanla-meli@uni-kassel.de
Universität Kassel, FB 18 Naturwissenschaften, Institut für Biologie
FG Ökologie, Heinrich-Plett-Str. 40, D-34132 Kassel, Germany

Abstract — Among fungal isolates from seeds of *Diospyros crassiflora*, one showed cultural and microscopic features of *Pestalotiopsis* species. DNA-sequence comparison and phylogenetic analyses using nucleotide sequences of internal transcribed spacer (ITS1-5.8S-ITS2) and the portion of nuclear large subunit (nuc-LSU) rDNA identified it as *Pestalotiopsis theae*. This finding indicated that *Pestalotiopsis theae,* a common pathogen that is often an endophyte or saprobe, may also be seminicolous.

Key words — acervuli, conidia, ribosomal RNA, seminicolous fungi

Introduction

Species of *Pestalotiopsis* Steyaert (*Xylariales*, *Xylariomycetidae*) form a cosmopolitan complex of fungi, which are economically important as agents of plant diseases (Chakraborty et al. 1994, Tuset et al. 1999, Nagata et al. 1992, Koh et al. 2001) and producers of pharmaceutical substances (Li et al. 1996, Strobel 2002). Besides the parasitic lifestyle, *Pestalotiopsis* species are also endophytes on living leaves and twigs but some are also saprobes often isolated from dead plant matter and even soil (Agarwal & Chauhan, 1988) and in plant debris (Osono & Takeda 2000).

In the Mbalmayo Forest Reserve, Cameroon, investigations were carried out to determine the diversity of fungi growing on seeds, termed seminicolous, of *Diospyros crassiflora* Hiern (*Ebenaceae*). Fungal isolates obtained were identified on the basis of cultural characteristics and molecular analysis. In addition to some *Trichocomataceae* species commonly known to be seminicolous — such as *Penicilliopsis clavariiformis* Solms and *Penicillium* spp. — one isolate had culture characteristics and micromorphology that related it to *Pestalotiopsis*. The aim of this study was to identify this isolate and to demonstrate the seminicolous character of *Pestalotiopsis*.

Materials and methods

Isolation, culture, and microscopic examination

During the fruiting period, unripe and ripe fruits of *D. crassiflora* were collected in the Mbalmayo Forest Reserve, Cameroon (Douanla-Meli 2007). Fresh seeds were removed from fruit, were surface-sterilized by dipping in 70% ethanol for 10 minutes, rinsed in deionised water, then were transversally divided. With aseptic scalpel, pieces of kernel (3 mm long) were cut up and placed on MYP (Malt Yeast Agar) medium containing 1% Tetracycline for avoiding bacterial growth, and incubated at room temperature. The hyphal tips emerging from seed pieces were cut and placed in new MYP Petri dishes without antibiotics. Pure colonies were subcultured on MYP and synthetic low-nutrient agar (SNA, Nirenberg 1976) in the light at room temperature and with 12 hours cool, white, fluorescent light and 12 hours of darkness at 25 °C. Microscopic features were studied from colony grown on MYP, mounted in 5% KOH. Conidia were photographed using Color View I Digital Camera. Colour terms in parentheses are those of Kornerup & Wanscher (1978).

DNA isolation and phylogenetic analyses

Genomic DNA was extracted from 5 days old culture grown at 25 °C on MYP using the CTAB method (Gardes & Bruns 1993). The primer pairs ITS1f/ITS4 (White et al. 1990) and LR0R/LR5 (Vilgalys & Hester 1990) were used for both PCR and sequencing of ITS1-5.8S-ITS2 and the portion of nuc-LSU rDNA respectively as described in Douanla-Meli et al. (2005) and Douanla-Meli & Langer (2008). New sequences have been deposited in GenBank with accession numbers EU833969 (nuc-LSU) and EU833970 (ITS). Additional sequences of *Pestalotiopsis* and allied species were retrieved from GenBank, the names and accession numbers are recorded on phylogenetic trees. The sequences were aligned in ClustalX (Thompson et al. 1977). Phylogenetic analyses were executed in PAUP* 4.0b10 (Swofford 2002) and used maximum likelihood (ML) with appropriate model determined by MrModeltest 2.2 (Nylander 2004). Tree topologies were tested with Kishino-Hasegawa (K-H) (Kishino & Hasegawa 1989) and Shimodaira-Hasegawa (S-H) (Shimodaira & Hasegawa 1999) maximum likelihood examinations. Pair differences among sequences were estimated using the "pairwise base differences" option in PAUP*.

Results and discussion

Phylogenetic analyses

The ITS and nuc-LSU rDNA sequences of the isolate DMC 698a had 559 and 910 bp respectively. In BLAST searches the ITS sequence showed 100% similarity with *P. theae* (Sawada) Steyaert strain PSHI2004Endo80 and

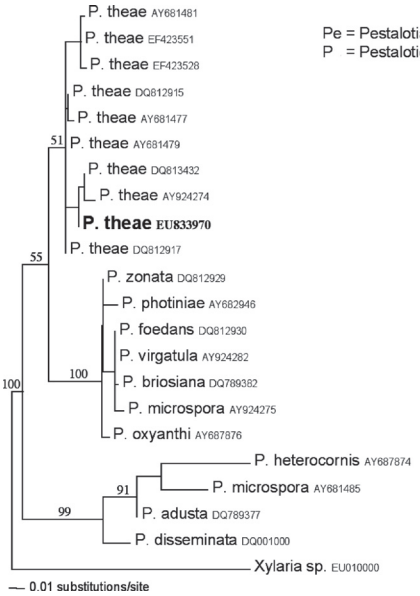


FIGURE 1. Phylogram of one of the 12 MLTs resulting from analyses of ITS1-5.8S-ITS2 sequences of *Pestalotiopsis* spp. –In L 2316.49095. Bootstrap values >50% are indicated above branches. In boldface is the isolate DMC 698a from seeds of *Diospyros crassiflora*. Tree was rooted to *Xylaria* sp.

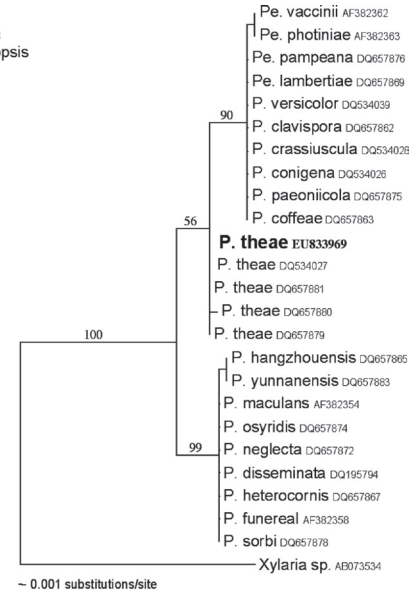
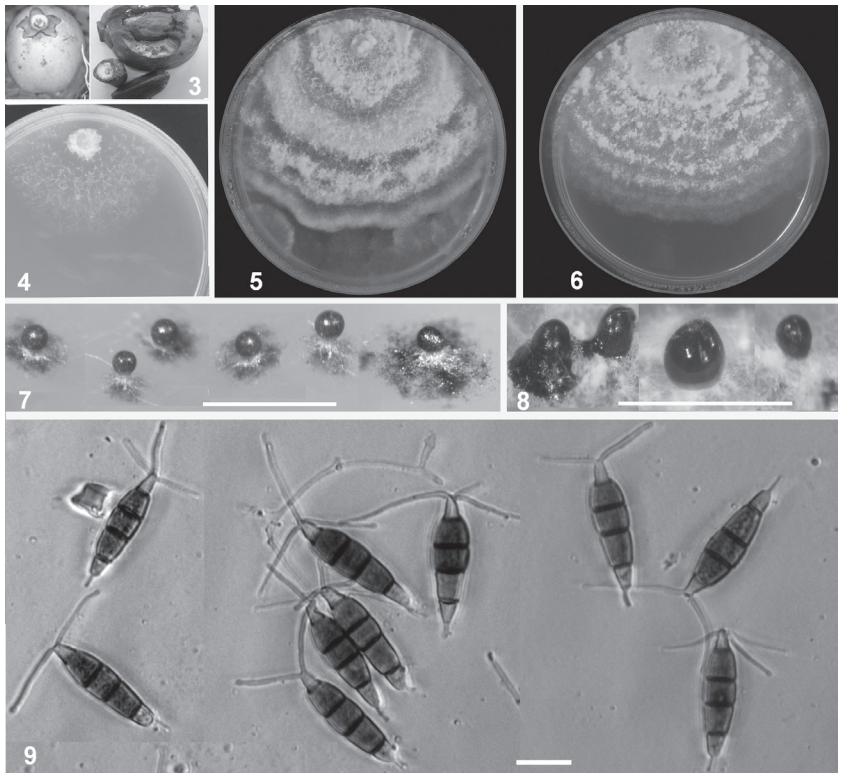


FIGURE 2. Phylogram of a single MLT resulting from analyses of nuc-LSU rDNA sequences of *Pestalotiopsis* and *Pestalotia* spp. –In L 1532.28485. Bootstrap values >50% are indicated above branches. In boldface is the isolate DMC 698a from seeds of *Diospyros crassiflora*. Tree was rooted to *Xylaria* sp.

the nuc-LSU had 100% similarity with *P. theae* strains PSHI2001path099, PSHI2004Endo46, and PSHI2001path205. Both ITS and nuc-LSU sequences of DMC 698a had 0–2% pair base differences with *P. theae* sequences from GenBank. The ITS dataset had 22 OTUs and included 520 characters after exclusion of ambiguous regions. The ITS1 regions were more ambiguously aligned, and had numerous indels than the ITS2 regions. The nuc-LSU dataset of 25 OTUs had 843 characters easily aligned, with a single indel at position 665. MrModeltest 2.2 suggested (HKY+I+G) and (GTR+I) as best-fit models for ITS and nuc-LSU datasets respectively. ML analyses of ITS dataset resulted in 12 most parsimonious likelihood trees (MLTs) of –In L 2316.49095. The best tree determined by K-H and S-H tests ($p < 0.05$) is presented (FIG. 1). ML analyses of LSU dataset resulted in a single MLT of –In L 1532.28485 (FIG. 2). Analyses of both datasets yielded well-resolved topologies delimiting a *P. theae* group, in which nested the isolate DMC 698a from seeds of *D. crassiflora*.



Figures 3-9. Fig. 3. Fruits and seeds of *Diospyros crassiflora*. Figs. 4-6. Colonies of *Pestalotiopsis theae*. 4. On SNA after 3 d. 5. On MYP at 25 °C with 12 hours cool, white, fluorescent light and 12 hours of darkness after 10 d. 6. On MYP in light at room temperature (20 °C) after 10 d. Fig. 7. Acervuli on SNA. Fig. 8. Acervuli on MYP. Fig. 9. Conidia. Bar = 1 mm for 7-8, 10 µm for 9.

Cultural and microscopic characteristics

Optimum temperature for growth 25–30°C. Colony radius after 3 d higher on SNA (27 mm) than MYP (25 mm) and growth subsequently slowing on MYP. Colonies grown on MYP growing faster at 25°C with 12 hours cool, white, fluorescent light and 12 hours of darkness than at room temperature (20 °C), white, floccose cottony, dense and thick, forming marked concentric rings with a barraging aspect, sometimes granulose on old rings due to highly intricate to aggregated hyphae, ultimate margin weakly fibrillose (Figs. 5–6), filling the Petri plate (85 mm) within 10–11 d, production of black (5E4) acervular conidiomata beginning by development of grayish white bands on colony ridges after 14 d, acervuli maturing within 4 d. Colonies grown on SNA pellicular, loose, producing abundant aerial mycelium (Fig. 4), not concentric,

later forming slender mycelial strands, filling the Petri plate (85 mm) within 9 d, acervular conidiomata formed earlier, after 7 d, and rather dispersed on a single, vague pseudoring, without grayish bands.

Acervuli on SNA (Fig. 7) isolate, very scattered, dark brown (6F8) to black (5E4-16G2), slimy and shiny, ampulliform, stipitate on the felted mycelium mixed with crystal-like elements, 200–250 µm in diameter and up to 350 µm high. On MYP, acervuli enfolded with water drops and mycelium until maturity, isolated to mostly concrete, larger, up to 350 µm in diameter. Conidia 23–31 × 5–7 µm, mean 25 × 6 µm, long fusiform, straight or rarely curved, five-celled (Fig. 9), not constricted, including three yellowish brown (5E8- 5D4) to dark brown (6F6) concolorous median cells, with dark bands at the septa. Median cells from the apex 5–7 × 5.5–6 µm, 5–7 × 6 µm, 5–8 × 5.5–6.5 µm respectively. Apical and basal cells hyaline, yellowish (2A2) to olivaceous (3F3). Apical cell conical, 3–5 × 3 µm and basal cell 4–5 × 2.5–3 µm. Appendages appearing at the apex and base, apical appendages 2–3, commonly 3 and rarely 4, 25–40 µm long with spheroidal tip, basal appendage 3–5 µm long.

Identification and ecology

Analyses of ITS and nuc-LSU rDNA sequences supported the placement in *Pestalotiopsis* of the strain DMC 698a isolated from seeds of *D. crassiflora*, as well as its assignment to *P. theae*. Species of *Pestalotiopsis* are usually differentiated on the basis of conidia characteristics such as size, septation, pigmentation, presence or absence and number of appendages (Nag Raj 1993). Conidial characteristics recorded for the strain DMC 698a covered the range for *P. theae*. For instance, the 5-celled conidia of the strain DMC 698a have three intermediate brown or yellowish brown concolorous cells, commonly 3 apical appendages knobbed at the extremities, the features segregating *P. theae* from the closely related species *P. fici* Steyaert, *P. annulata* (Berk. & M.A. Curtis) Steyaert and *P. jesteri* Strobel et al. (Guba 1961, Strobel et al. 2000).

Like other allied species, *P. theae* is not host-specific (Jeewon et al. 2004, Hawksworth 2005, Tejesvi et al 2007, Wei et al 2007) and has been isolated from a number of unrelated hosts. It is commonly a pathogen, known as causal agent of gray blight disease on leaves of *Camellia sinensis* (L.) Kuntze (Chakraborty et al. 1994, Mordue & Holliday 1971, Koh et al. 2001), and causes also leaf necrosis in many tropical fruit and crop plants, e.g. *Cocos nucifera* L., *Diospyros kaki* L.f., *Elaeis guineensis* Jacq., *Mangifera indica* L., *Psidium guajava* L., *Theobroma cacao* L. Nevertheless, *P. theae* is not consistently pathogenic. It has also been isolated from healthy cambium of *Cinnamomum iners* Blume (Woprapong et al. 2003), thus as endophyte. The behaviour of *P. theae* against its host may vary upon environmental conditions, therefore a given strain of *P. theae* can be pathogenic or endophytic on different hosts. Support to this allegation was

provided by ITS analyses in which both pathogenic and endophytic strains of *P. theae* clustered in one clade (Wei et al. 2007). *Pestalotiopsis* species yet isolated from *Diospyros* L. are usually pathogens. Mostly leaves are attacked, like *P. versicolor* (Speg.) Steyaert reported from Madhya Pradesh, India, to cause foliage disease of *D. melanoxylon* Roxb. (Harsh et al. 1987), and similarly, *P. theae* reported as pathogenic fungus of leaf blight in sweet persimmon (*D. kaki*) in Spain (Tuset 1999).

Pestalotiopsis generally colonizes leaf, twig and bark tissue of the host plants. Only a few species have been yet isolated from fruits, viz. *P. heterocornis* (Guba) Y.X. Chen and *P. zonata* (Ellis & Everh.) G.C. Zhao & N. Li from *Podocarpus macrophyllus* (Thunb.) Sweet (Liu et al. 2007, Wei et al. 2007), and *Pestalotiopsis psidii* (Pat.) Mordue responsible for scabby fruit canker of *Psidium guajava* (Keith et al. 2006). In this study *P. theae* (strain DMC 698a) was isolated for the first time from fruits of *D. crassiflora*. Moreover, *P. theae* was isolated for the first time from seeds. This finding gave evidence that this fungus may also be seminicolous. *P. theae* co-occurred on seeds of *D. crassiflora* with an unspecified *Penicillium* and the common seminicolous fungus *Penicilliopsis clavariiformis*. For *Penicilliopsis clavariiformis*, it was stipulated that it infects the seeds while they are still on the plant (Samson & Seifert 1985), this is presumably also valid for *P. theae*. On green unripe as well as on yellow ripe fruits of *D. crassiflora*, neither spots nor corky lesions indicating disease symptoms were observed.

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