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RESEARCH ARTICLE

First report and characterization of *Fusarium circinatum,* the causal agent of pitch canker in Brazil

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ABSTRACT

Pitch canker is one of the most important diseases of pine trees worldwide, including South America. The causal agent of this disease is *Fusarium circinatum*, a member of the *Gibberella fujikuroi complex* (GFC). In South America, the species is reported from Colombia, Uruguay and Chile, but is considered a quarantine organism in Brazil. In this study we characterized isolates obtained from symptomatic pine seedlings intercepted in a nursery in Santa Catarina State using phylogenetic analyses, crossings and morphological markers. The Brazilian isolates grouped with reference material in an unique clade and formed fertile perithecia when crossed with reference tester strains. The detailed characterization given here and the availability of tester strains will allow for reliable identification and support monitoring of this important plant pathogen.
Key words: *Pinus*, *Gibberella fujikuroi* species complex, mating population, molecular phylogeny.

INTRODUCTION

International trade has long been recognized as a major conduit by which nonindigenous plant pests arrive in Brazil and other countries. Invasive plant pathogens have dramatically affected the crops productivity and function of natural and agricultural ecosystems. Pitch canker of pine (*Pinus* spp.) is caused by *Fusarium circinatum* Nirenberg & O'Donnell and is frequently associated with high levels of mortality in nurseries and on mature trees, resulting in significant economic losses (Wingfield et al., 2008). The disease was first discovered in the southeastern United States (Hepting & Roth, 1946; Gordon et al., 2001) and the causal agent described as *F. subglutinans* f. sp. *pini* (Correll et al., 1991). The pathogen was then recorded in numerous countries worldwide, including Mexico, Colombia, Chile and Uruguay (Britz et al., 2001; Wingfield et al., 2002; Alonso & Bettucci, 2009; Steenkamp et al., 2012). *F. circinatum* is known to infect more than 50 species of pines (Wingfield et al., 2008). Symptoms of infection in nurseries include damping-off and wilting of seedlings. On mature trees, pitch canker is characterized by heavy exudation of resin at the site of infection (Wingfield et al., 2008) although other plant parts such as roots, shoots, female flowers and mature cones, seed and seedlings can also be affected (Viljoen et al., 1994).

This fungus is a member of the *Gibberella fujikuroi complex* (GFC) and is known to reproduce both sexually and asexually. The sexual form *Gibberella circinata* is cross-fertile under laboratory conditions and represents a distinct mating population (MP-H) in the GFC (Britz et al., 1998; 1999). Morphological characters like the production of sterile coiled hyphae are not consistent and therefore reliable identification must be complemented by a phylogenetic analysis or laboratory crosses (Leslie & Summerell, 2006). Here we report the occurrence of *Fusarium circinatum* during an interception in Brazil and characterize local strains by means of phylogeny, crossings, morphological characters and pathogenicity tests.

MATERIALS AND METHODS

Cultures and culture conditions

The present study included strains isolated from diseased *Pinus taeda* seedlings collected in a nursery in Santa Catarina State, Brazil and reference strains of *F. circinatum* (MRC 7488 - MAT1-1; MRC 6213 - MAT1-2; NRRL 25331 - Type strain). *Pinus taeda* seedlings produced from imported seeds showed symptoms of wilting and some were killed by the disease, and isolates obtained from symptomatic plants were initially identified as *Fusarium subglutinans* (Grigoletti Júnior et al., 2006). All evaluated strains were used as single spore cultures and are deposited as spore suspension in 15% glycerol at -70ºC at CML - Coleção Micológica de Lavras, Universidade Federal de Lavras (Table 1).

Morphological and cultural studies

Potato dextrose agar (PDA) was used to evaluate characters of the colony and calculate the growth rate.

TABLE 1 - Strains of *Fusarium circinatum* and GenBank accession numbers of *Fusarium* spp. of the *Gibberella fujikuroi* species complex GFC used to generate the phylogram in this study.

Species	CML	Other code [a]	Host/Substrate	Origin	*tef1*	*cmd*
F. circinatum	790	MRC 7488 Mat1-1, FGSC 9022, KSU H-10847	*Pinus patula*	South Africa	KF597824	KF597829
F. circinatum	791	MRC 6213 Mat1-2, FGSC 9023, KSU H-10850	*Pinus patula*	South Africa	KF597823	KF597828
F. circinatum T	405	NRRL 25331, BBA 69720, MRC 7869, E303; Mat1-1	*Pinus radiata*	USA	AF160295	AF158348
F. circinatum	2128	E380	*Pinus taeda*	Brazil SC	KF597825	KF597830
F. circinatum	2367	E379	*Pinus taeda*	Brazil SC	KF597822	KF597831
F. circinatum	2368	E542	*Pinus taeda*	Brazil SC	KF597827	KF597832
F. circinatum	2369	E543	*Pinus taeda*	Brazil SC	KF597826	KF597833
F. acutatum		NRRL 13308	*unknow*	India	AF160276	AF158329
F. anthophilum		NRRL 13602	*Hippeastrum* sp.	Germany	AF160292	AF158345
F. concentricum T		NRRL 25181	*Musa sapientum*	Costa Rica	AF160282	AF158335
F. dlamini T		NRRL 13164	*Zea mays*	South Africa	AF160277	AF158330
F. fujikuroi		NRRL 13566	*Oryza sativa*	Taiwan	AF160279	AF158332
F. globosum		NRRL 26131	*Zea mays*	South Africa	AF160285	AF158338
F. guttiforme T		NRRL 22945	*Ananas comosus*	England	AF160297	AF158350
F. inflexum		NRRL 20433	*Vicia faba*	Germany	AF008479	AF158366
F. napiforme T		NRRL 13604	*Pennisetum typhoides*	South Africa	AF160266	AF158319
F. nygamai T		NRRL 13448	*Sorghum bicolor*	Australia	AF160273	AF158326
F. oxysporum		NRRL 22902	*Pseudotsuga menziesii*	USA	AF160312	AF158365
F. phyllophilum		NRRL 13617	*Dracaena deremensis*	Italy	AF160274	AF158327
F. proliferatum		NRRL 22944	*Cattleya* sp.	Germany	AF160280	AF158333
F. pseudocircinatum T		NRRL 22946	*Solanum* sp.	Ghana	AF160271	AF158324
F. pseudonygamai T		NRRL 13592	*Pennisetum typhoides*	Nigeria	AF160263	AF158316
F. sacchari		NRRL13999	*Saccharum officinarum*	India	AF160278	AF158331
F. sterilihyphosum T	414	NRRL 25623	*Mangifera indica*		AF160300	AF158353
F. subglutinans		NRRL 22016	*Zea mays*	USA	AF160289	AF374046
F. thapsinum		NRRL 22045	*Sorghum bicolor*	South Africa	AF160270	AF158323
F. tupiense T	262	NRRL 53984	*Mangifera indica*	Brazil	DQ452859	
F. verticillioides		NRRL 28899	*Zea mays*	Germany	AF273318	AF158315

[a] Culture collection abbreviations: CML, Coleção Micológica de Lavras, Universidade Federal de Lavras, Lavras, Brazil; NRRL, The Agriculture Research Service Culture Collection, National Center for Agricultural Utilization Research, USDA/ARS, Peoria, Illinois, USA; BBA, Biologische Bundesanstalt für Land- und Forstwirtschaft, Institut für Virologie, Mikrobiologie und Biologische Sicherheit, Berlin, Germany; E, Incaper Culture Collection, Plant Pathology Laboratory, Vitória ES, Brazil; FGSC, Fungal Genetics Stock Center, Kansas City, Missouri, USA; KSU, Kansas State University, Manhattan, Kansas, USA; MRC, Medical Research Council, Tygerberg, South Africa. T= Type strains.

Morphological characteristics of the strains were observed on synthetic nutrient-poor agar (SNA). Cultures were incubated for 10 to 14 days at 20°C in a 12h photoperiod under near UV and white fluorescent bulbs. The strains were cultivated at 20°C on PDA in the dark for determination of growth rates and color. Colony diameters were measured on three plates of each culture after 4 days of incubation (Nirenberg & O'Donnell, 1998; Leslie & Summerell 2006).

Sexual compatibility and laboratory crosses

A *MAT1-1* allele fragment was amplified using the primer set Gfmat1a and Gfmat1b, and a *MAT1-2* allele fragment was amplified with the Gfmat2c and Gfmat2d primers (Steenkamp et al., 2000). Strains of opposite mating types were intercrossed and also with tester type strains of *Fusarium circinatum* (MRC 7488 - MAT1-1; MRC 6213 - MAT1-2) (Leslie & Summerell, 2006). Strains were grown in tubes with complete medium for one week at 20°C under 12-h photoperiod to be used as male parents. Conidial suspensions were prepared by adding 2 mL sterile distilled water. Tester strains used as female parents were grown on carrot agar (CA) plates for one week at 25°C in the dark and fertilized by adding 2 mL of the conidial suspensions of the Brazilian strains onto the surface of

the CA plates, spreading and mixing vigorously all over the surface (Klittich & Leslie, 1988; Leslie & Summerell, 2006). The plates were incubated upright at 20°C under 12-h photoperiod. All crosses were repeated twice. Crosses were evaluated weekly during six weeks for formation of perithecia and ascospore exudation. A cirrus of ascospores was collected from three perithecia of each fertile cross, suspended in sterile water, and spread on the surface of 2% water agar in a Petri dish. Plates were incubated overnight at 25ºC in the dark and checked for ascospore germination (Lima et al., 2012). Special care was taken to avoid transfer of conidia with ascospores.

DNA extraction, PCR amplification and sequencing

Monospore cultures were grown in complete medium broth (CM; Correll et al., 1987), harvested by filtration, and DNA was extracted with a standard CTAB protocol (Leslie & Summerell, 2006). Portions of the *cmd* gene were amplified from representatives of *F. circinatum* and *F. sterilihyphosum* with primers CL1 (forward; 5'-GAR TWC AAG GAG GCC TTC TC-3') and CL2A (reverse; 5'-TTT TTG CAT CAT GAG TTG GAC-3') using the amplification conditions described by O'Donnell et al. (2000). Portions of the *tef1* gene were amplified by using primers Ef-1 (forward; 5′-ATG GGT AAG GAG GAC AAG AC-3′) and Ef-2 (reverse; 5′-GGA AGT ACC AGT GAT CAT GTT-3′) with the amplification conditions of O'Donnell et al. (1998). PCR products were stained using Gel-Red stain (Biotium), separated by electrophoresis in 1% agarose gels, and visualized under UV light (312 nm). PCR amplified fragments were purified with a Gen Elute PCR cleanup kit (Sigma-Aldrich) and sequenced in both directions on an automated MEGA BACE 1000 capillary sequencer (GE Healthcare).

Sequence alignment and phylogenetic analyses

Consensus sequences were assembled from forward and reverse sequences using SeqAssem ver. 07/2008 (SequentiX - Digital DNA Processing). Additional sequences of other species of the *Gibberella fujikuroi complex* were obtained from Genbank (Altschul et al., 1997). Sequences were aligned using ClustalW as implemented by Mega 5 (Tamura et al., 2011). Maximum Parsimony (MP) phylogenetic analysis was performed on Mega 5 (Tamura et al., 2011) using the Close-Neighbor-Interchange algorithm with search level 1, and ten initial trees were obtained with the random addition of sequences. Clade support was inferred from 1000 bootstrap replications. All positions containing gaps were eliminated from the analysis (Phoulivong et al., 2010). Sequences of *F. oxysporum* NRRL 22902 and *F. inflexum* NRRL 20433 were used to root the trees based on prior analyses of the GF-C.

Pathogenicity tests

The pathogenicity tests were conducted with two of the Brazilian isolates (CML 2128, CML 2369), and one strain of *Fusarium tupiense* (CML 262) as control. Seedlings of *Pinus patula* were grown in the greenhouse at 26±3°C under natural conditions, and then transplanted to 5 L pots containing a 50:50 mixture of soil and a commercially available substrate (Plantmax). Inoculum was prepared by culturing each isolate on 6-cm plastic Petri dishes containing Malt Extract 2% for 1 week at 25°C in the dark, after which agar plugs 6 mm in diameter were excised from the growing edge of the colony for inoculation. Wounds were made in the upper third of the stem using a fine steel needle. The plugs with mycelium were placed on the wounds and covered with plastic film to prevent desiccation (Roux et al., 2007). Pots were then placed in the greenhouse under natural photoperiod at 26±3°C for 5 weeks. Treatments were arranged in a completely randomized design with five replications. Not inoculated plants served as a negative control. Assessments were made weekly, during 8 weeks. Inoculation experiments were conducted twice in time.

RESULTS

Strains obtained from *Pinus* seedlings in Santa Catarina were identified as *Fusarium circinatum* based on phylogeny, laboratory crosses and morphological features. Amplified DNA sequences of the *tef1* and *cmd* gene fragments were aligned with the sequences of reference strains and other species of the GFC available from GenBank. In all tree topologies generated by Maximum Parsimony (MP), in separate analyses of *cmd* and *tef1* as well as in the combined analysis, isolates from *Pinus* in Brazil clustered together with the type strain of *F. circinatum* in a strongly supported clade (99%) (Figure 1).

The presence of both mating idiomorphs from the Brazilian isolates was confirmed by amplification of the MAT loci. Two isolates belong to *MAT1-1* mating type idiomorph (CML 2128, CML 2369), whereas two others contained the *MAT1-2* mating type idiomorph (CML 2367, CML 2368). Perithecia were observed after four weeks in all crosses between the individual tester strains of MP-H (MRC 7488, *MAT1-1*; MRC 6213, *MAT1-2*) and the strains of *Fusarium* associated with *Pinus* in Brazil (Figure 2E). The morphological characteristics of perithecia, ascus and ascospore were consistent with those described in the literature (Nirenberg & O'Donnell, 1998; Britz et al., 2002). About 80-90% of ascospores germinated within 24 h of transfer to water agar.

Cultures showed sterile hyphae in the aerial mycelium, monophialides and polyphialides with two or more openings on proliferating conidiophores, and produced obovoid conidia aggregated in false heads in the aerial mycelium, mostly non-septate or occasionally with one septum. Macroconidia were produced in sporodochia, pale orange in color, relatively slender with no significant curvature and usually tri-septate (Figure 2A-D). No chlamydospores were observed. Colonies grown on PDA had a lanose aspect, with white or slightly pink aerial

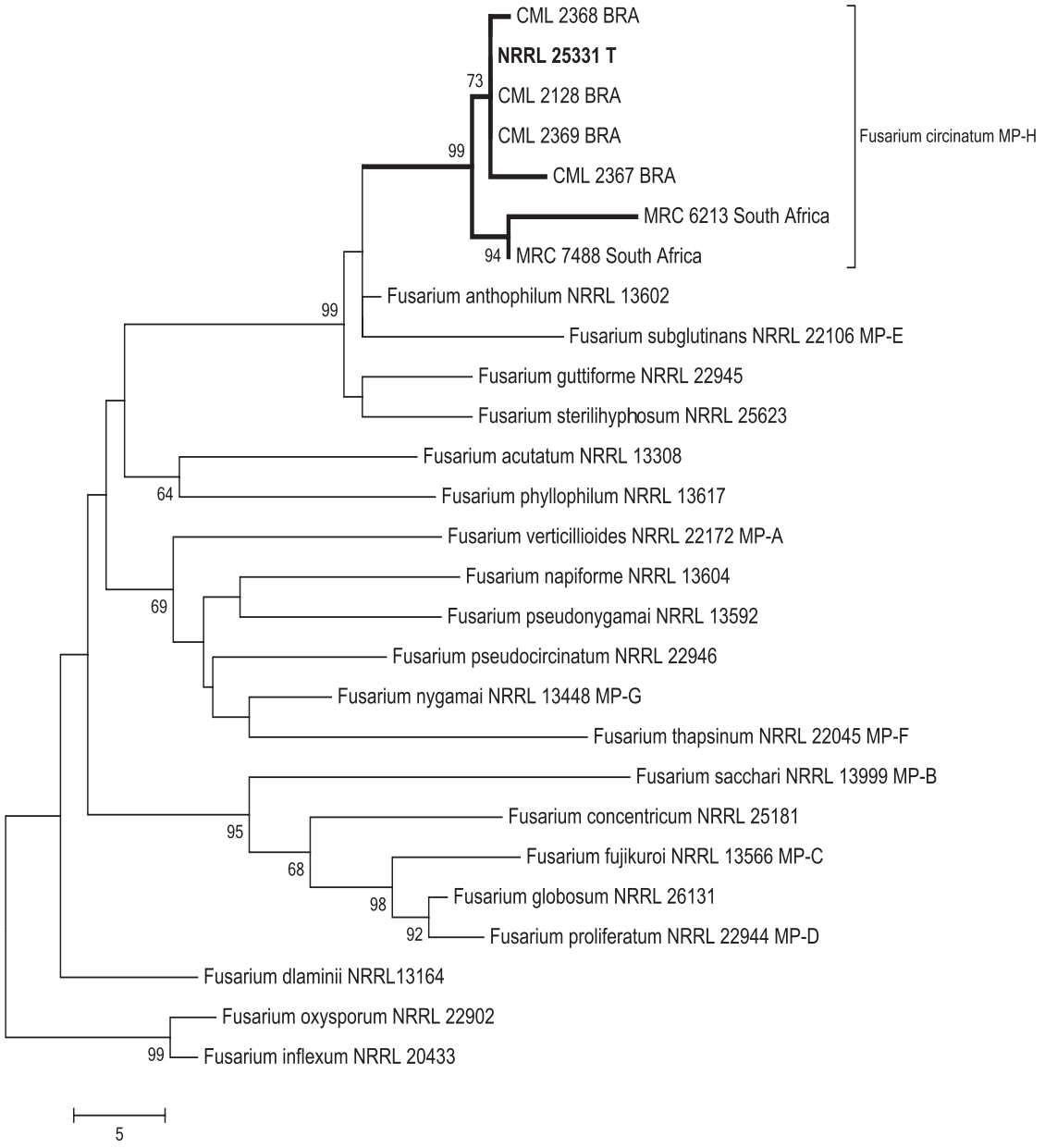

FIGURE 1 - One of 11 maximum parsimony phylograms inferred from the combined datasets of *cmd* and *tef1* showing the relationship of isolates associated with pitch canker of *Pinus* in Brazil with species in the *Gibberella fujikuroi* species complex (MP = *mating population*). Branch lengths are indicated by the scale at the bottom and bootstrap values (1000 replications) are above internodes. This tree is rooted to *F. oxysporum* and *F. inflexum*. Culture collection abbreviations: CML = Coleção Micológica de Lavras, Lavras, MG, Brazil; NRRL = National Center for Agricultural Utilization Research, Peoria, IL, USA; KSU = Kansas State University, Manhattan, KS, USA; MRC = Medical Research Council, Tygerberg, South Africa.

mycelium and appearing salmon colored from the underside of the plate, eventually with a violet pigment in the agar. The average mycelial growth rate was 5 mm/d at 20°C.

In the pathogenicity tests, isolates induced typical symptoms after about 21 days of inoculation. The infected seedlings showed discolored lesions with yellowing of needles evolving to burning and leaf fall. After 35 days, the seedlings showed symptoms of dieback in the upper part (Figure 2F). The isolate of *F. tupiense* didn't induce symptoms on inoculated plants. To fulfill Koch's postulates, the pathogen was re-isolated from inoculated plants and identified as *F. circinatum* based on morphology.

DISCUSSION

Results of phylogenetic analyses, mating compatibility tests, evaluation of morphological markers and pathogenicity tests showed that the fungus associated

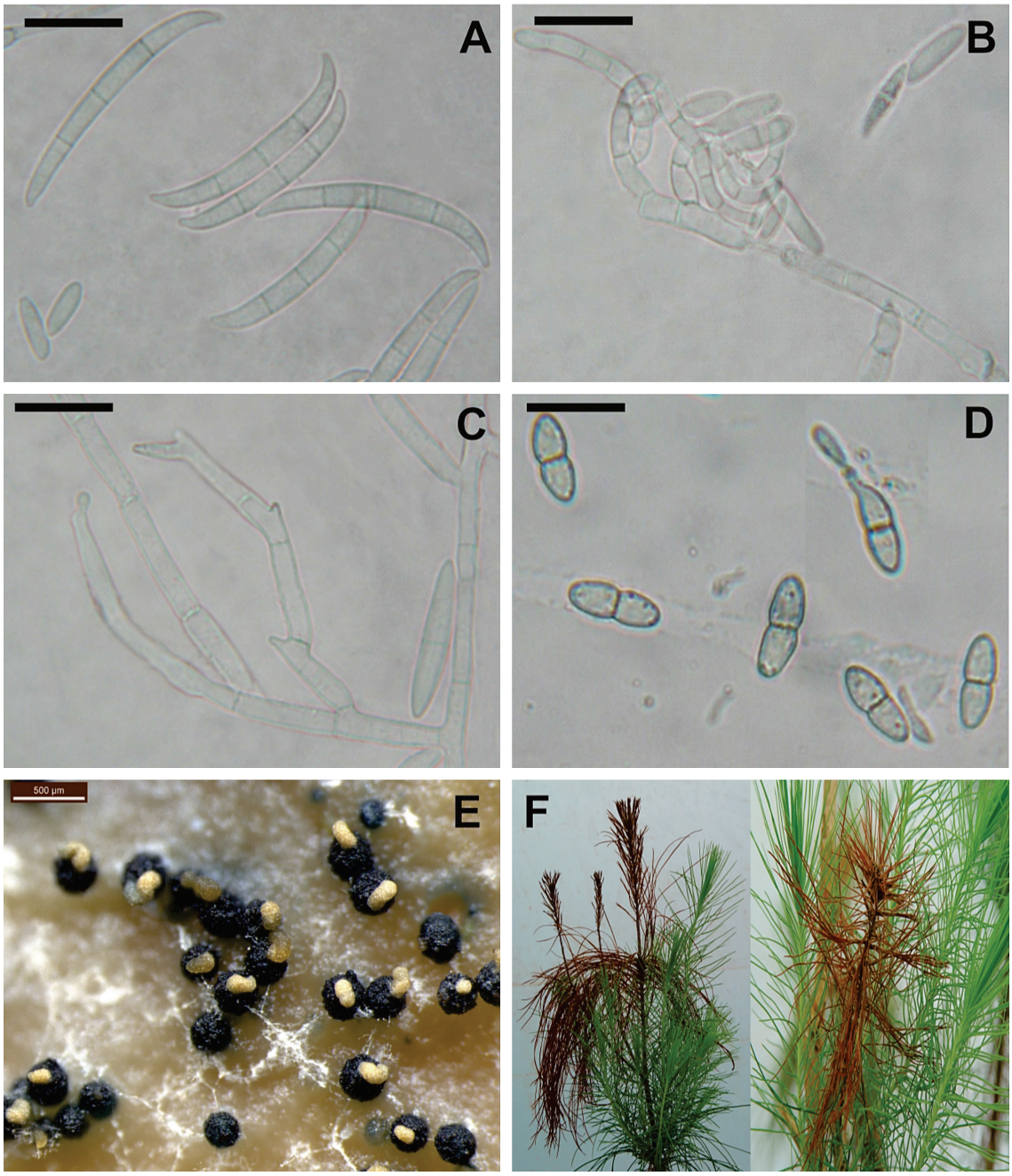

FIGURE 2 - Morphology of *Fusarium circinatum* and symptoms. **A**. Macroconidia, bar=20 µm; **B**. Sterile coils, bar=20 µm; **C**. Polyphialides, bar=10 µm; **D**. Ascospores, bar=15 µm; **E**. Perithecia with oozing ascospores; **F**. Inoculated *Pinus patula* seedlings showing dieback symptoms.

with diseased seedlings of *Pinus* in Brazil is *F. circinatum*. The application of different species concepts allowed for a reliable and consistent identification of this species, which is a member of the GFC, where morphological markers are scarce or sometimes even absent (Kvas et al., 2009; Lima et al., 2012). The species was first described as a special form of *F. subglutinans*, as it shares the main morphological features with this species, like the presence of polyphialides, production of conidia in the aerial mycelium only in false heads and the absence of chlamydospores (Correll et al., 1991; Nirenberg & O'Donnell et al., 1998). Morphological characteristics typical of *F. circinatum* like conidiophores on the erect aerial mycelium, polyphialides with more than two conidiogenous openings and sterile coils could be observed, but these characters are shared with species like *F. mexicanum, F. pseudocircinatum, F. sterilihyphosum* and *F. tupiense* (Nirenberg & O'Donnell, 1998; Britz et al., 2002; Lima et al., 2012).

All sexual crosses of strains from Brazil with mating tester strains of *F. circinatum* produced the sexual stage. These crosses between the individual tester strains of the MP-H from South Africa and strains from Brazil evidence that no reproductive isolation between both populations exists. Moreover, the presence of both MAT idiomorphs indicates that sexual reproduction may occur in Brazil. The sexual reproduction increases the genetic variability of the pathogen causing potential consequences for breeding programs and the management of this pathogen.

Pathogenicity tests revealed that strains recovered from symptomatic *Pinus patula* in Brazil were pathogenic to *P. patula*. The pathogen survives in soil and can be seedborne, so there can be extensive mortality of seedlings in planted and natural *Pinus* stands. Also, pitch canker is frequently associated with a reduction in growth volume and is often associated with significant economic losses (Viljoen et al., 1994; Storer et al., 1998; Wingfield et al., 2008). *Fusarium circinatum* was recently reported in association with grass species as symptomless hosts in the proximity of *Pinus* stands (Sweet & Gordon, 2012). The ability to survive in alternative hosts is relevant for establishment and subsequent dissemination of the pathogen to new areas. The movement of infested seeds could also explain the introduction of *F. circinatum* to new areas (Gordon, 2006).

In South America, pitch canker and its causal agent were reported previously in Chile, Uruguay and Colombia (Wingfield et al., 2002; Alonso & Bettucci, 2009; Steenkamp et al., 2012). We suggest that the pathogen was introduced to the nursery in Brazil on seeds from external sources. Rouging (eradication) of diseased material in nurseries may have prevented the spread of this fungus. No plants with symptoms of pitch canker were found in subsequent surveys for diseased plants in nurseries and plantations of *Pinus*. Nevertheless, introduction of this pathogen to new areas in recent years calls for strict monitoring and sanitation practices (OEPP/EPPO, 2005).

This study represents the first confirmed report *F. circinatum* in Brazil, which is considered a quarantine fungus and one of the most serious pathogens of *Pinus*. This complete characterization of the pathogen and available tester strains for laboratory crosses will allow for reliable identification and support future monitoring of this pathogen in Brazil. New surveys should be conducted in areas planted with *Pinus* in southern Brazil because the presence of the pathogen is a major threat to the commercial use of pines.

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