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Full Length Research Paper

Alternaria jacinthicola, a new fungal species causing blight leaf disease on water hyacinth [*Eichhornia crassipes* (Martius) Solms-Laubach]

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Water hyacinth (*Eichhornia crassipes*) causes environmental, agricultural and health problems in Mali. This is particularly severe in the District of Bamako and the irrigation systems of the "Office du Niger" area. During two years survey for fungal pathogens of water hyacinth infested areas, isolate Mlb684 was collected from diseased plant. This fungal isolate was identified as a potential mycoherbicide for sustainable management for water hyacinth. The aim of this study was to characterize isolate Mlb684. The characterization was based on a morphological description and a DNA sequence analysis. Various genes amplified from isolate Mlb684 were compared to those existing in Genbank. These genes were 18S ribosomal rDNA gene, ITS rDNA gene, elongation factor-1 alpha (EF1a) gene, calmodulin and actin genes. DNA sequence comparisons and morphological description provided enough evidences to show that isolate Mlb684 belonged to the *Alternaria* genus and was distinct from any other known *Alternaria* species. Based on these evidences, the new fungal isolate was called "*Alternaria jacinthicola* Dagno & M.H. Jijakli". A specimen culture has been deposited in the Gembloux Agro Bio Tech Plant Pathology unit fungal collection, with Mlb684 reference and in the Industrial Fungal and Yeast Collection (BCCM/MUCL, Belgium) under the accession number: MUCL 53159 and all DNA sequences were deposited in GenBank (NCBI).

Key words: ITS rDNA, 18S rDNA, actin, calmodulin, elongation factor, genetic characterization, *Alternaria jacinthicola, Eichhornia crassipes,* water hyacinth.

INTRODUCTION

Water hyacinth (*Eichhornia crassipes*) has spread throughout Africa causing widespread problems to millions of users of water bodies and water resources. The plant affects irrigation, water flow, water use, and navigation; it also poses a health risk by enabling the breeding of mosquitos, bilharzias, and other human parasites (Dagno et al., 2007). Biocontrol has been considered as the most adequate control strategy against water hyacinth (Charudattan, 2005). Among possibilities offered by the biocontrol management, fungal pathogens could be an efficient control tool against this aquatic weed. Several research groups have identified promising microbial fungal agents notably *Alternaria eichhorniae* Nag Raj & Ponnappa and *Alternaria alternata* (Fr.) Keissler that might be developed and used as mycoherbicides (El-Morsy, 2006; Babu et al., 2002; Shabana, 1997). Research on fungal pathogens of water hyacinth began in 2006 in Mali and led to the isolation and identification of *Gibberella sacchari* Summerell & J.F. Leslie (isolate Mln799), *Cadophora malorum* (Kidd & Beaumont) W. Grams (isolate Mln715) and isolate Mlb684 (Dagno et al., 2011a). Among the 3 selected fungi, an unusual fungus, isolate Mlb684 applied in

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Table 1. PCR primers for amplification of genes of *Alternaria* sp. isolate Mlb684.

Primers names	Sequences
[1]ITS1	5'TCCGTAGGTGAACCTGCGG3'
[1]ITS4	5'TCCTCCGCTTATTGATATGC3'
[1]ITS5	5'GGAAGTAAAAGTCGTAACAAG3'
[1]NS1	5'GTAGTCATATGCTTGTCTC3'
[1]NS2	5'GGCTGCTGGCACCAG…..TGC3'
[2]EF1-728F	5'CATCGAGAAGTTCGAGAAGG3'
[3]TEF1LLErev	5'AATTTGCAGGCAATGTGG3'
[2]CAL-228	5'GAGTTCAAGGAGGCCTTCTCCC3'
[2]CAL-737R	5'CATCTTTCTGGCCATCATGG3'
[2]ACT-783R	5'TACGAGTCCTTCTGGCCCAT3'
[2]ACT-512F	5'ATGTGCAAGGCCGGTTTCGC3'

[1]White et al. (1990), [2]Carbone and Kohn (1999), [3]Jaklitsch et al. (2005).

unrefined *Carapa procera* (L) oil and refined palm oils caused 87 to 90% of disease severity on water hyacinth 6 weeks after treatment respectively (Dagno et al., 2011b).

In 2007, the Industrial Fungal and Yeast Collection (BCCM/MUCL, Belgium) identified isolate Mlb684 as *Alternaria* sp. with reference BCCM/MUCL DIV/07-119C. However, morphological characters displayed by this isolate including the pattern and sporulation structure of this isolate hardly match with those currently used to describe known species in the genus *Alternaria* (E.G.S. characteristics, as described in the literature: Simmons, 2004; Simmons, 1999; Simmons and Roberts, 1993). It was therefore assumed that this isolate could belong to a novel species of *Alternaria*. Two *Alternaria* species were until now reported on water hyacinth. *A. eichhorniae* and *A. alternata* were recognized as virulent pathogens on *E. crassipes* species. They are best known as the causal agent of leaf blight disease on water hyacinth in Egypt and India (Shabana et al., 1995; Aneja and Sing, 1989).

The present study was designed to provide a taxonomic position of the isolate Mlb684 at the species level, using morphological characterization and DNA sequence comparisons.

MATERIALS AND METHODS

Fungal isolation and specimen collection

Infected parts of water hyacinth (petioles and leaves) were collected from the River Niger in the District of Bamako, the lake of Sebougou in Segou, and the central collector of Niono with GPS coordinates "12° 40' N, 7° 59' W" ; "13° 26' N, 6° 15' W" and "14° 15' N, 5° 59' W" respectively. Samples in clean plastic bags were brought to the laboratory and then stored at 4°C until examined. Stored plant parts were scrubbed under running water to remove surface debris, dissected into small segments (approximately 1 × 1 cm), and surface-sterilized by sequential immersion in 96% ethanol for 30 s and then in 14% hypochlorite for 30 s. The segments were rinsed in sterilised water for 1 min. Surface-sterilised segments (4 segments/plate) were plated on potato dextrose agar (PDA, Merck, Darmstadt, Germany) supplemented with 5 ppm streptomycin. Three plates were used for petiole and limb of water hyacinth plant. The plates were then incubated at 25°C for 5 to 7 days. Fungal mycelia that emerged from the plant fragments were isolated and pure cultures were obtained by the single-spore technique, cultures were then preserved at 4°C, for no more than 6 months, before use. Isolate Mlb684 was selected during a previous assay conducted to identify potential mycoherbicide for water hyacinth (Dagno et al., 2011a, b). Pure cultures were deposited in the Culture Collection of the Phytopathology Unit, Gembloux Agro Bio Tech (GxABT), University of Liege, Belgium. Duplicates of key isolates (specimens) were also deposited to the Industrial Fungal and Yeast Collection (BCCM^{TM}/MUCL - Louvain-la-Neuve, Belgium). For mass production, isolate Mlb684 was incubated on V8 agar during 2 weeks at 25°C and 16 h photoperiod.

Molecular analysis

A liquid culture from the isolate Mlb684 was performed in 100 ml of potato dextrose broth (PDB) in a 250 ml Erlenmeyer for 5 days. Pure DNA was obtained from the resulting culture using a quick DNA extraction method (Lefort and Douglas, 1999). Universal oligonucleotide primers (Table 1) targeting 5 fungal genes were used for PCR amplification. Primers ITS1, ITS4 and ITS5 (White et al., 1990) for the 18S and 28S rDNA sequence; primers NS1 and NS2 (White et al., 1990) for a partial 18S rDNA sequence; primers EF1-728F (Carbone and Kohn, 1999) and TEF1LLEre (Jaklitsch et al., 2005) for the elongation factor-1 alpha (EF1a); primers CAL-228 and CAL-737R (Carbone and Kohn, 1999) for calmodulin gene; and primers ACT-783R and ACT-512F (Carbone and Kohn, 1999) for actin gene. PCR was carried out using the KAPA2G Robust PCR (KappaBiosystems, Japan) and each PCR amplification was performed in 20 µl reaction mixture consisting of 10 µl Maxima Sybr Green qPCR Master Mix 2X (Fermentas), 2 µl each of the forward and reverse primers (10 µM), 1 µl cDNA template (1 ng/µl), and 5 µl PCR-grade water. The cycling conditions were: pre-incubation for 10 min at 95°C, followed by 40 cycles, each consisting of 30 s denaturing at 95°C, 40s annealing at 52°C, and 45 s elongation at 72°C, the last cycle ending with a final 10-min extension at 72°C.

Total genomic DNA was extracted according to Lefort and Douglas (1999), and the concentration of the resulting DNA was determined with an ND-1000 UV/Vis spectrometer (NanoDrop Technologies, Wilmington, DE USA) version 3.1.0. Oligonucleotide primers (Table 1) were used to amplify and sequence the internal transcribed spacer (ITS) regions (including the partial 18 S and 28 S DNA genes) and regions corresponding to the genes encoding elongation factor-1 alpha (EF1a), calmodulin, and actin. DNA sequences were edited by Fasteris SA, Geneva, Switzerland (Lefort and Douglas, 1999). Resulting DNA sequences were deposited in GenBank (NCBI, Bethesda, MD, USA) and compared using the similarity search tool Blast.

DNA sequences recovered from GenBank for species close to the *Alternaria* isolate Mlb684 were aligned by ClustalW2 (Chenna et al., 2003) and used to generate molecular phylogenies with an optimal neighbour-joining method (Myers and Miller, 1988). Trees were then drawn with the software Jalview (Waterhouse et al., 2009), in addition, a Bayesian analysis was run to this one.

Morphology

Isolate Mlb684 were grown on V8 agar and potato-carrot agar mediums under strictly defined incubation conditions (Simmons, 1992) and examined for characteristics of the sporulation apparatus and conidium morphology to confirm species identity and compare morphological characters. Morphological description was performed

Table 2. Comparisons of DNA sequences between *Alternaria* sp. isolate Mlb684 and the closest fungal for which sequences were available in Genbank.

Fragment size	Species and sources	Genbank accession numbers	Homology percentage
	Alternaria sp. (isolate MUCL 45333)	AY714488	100
ITS rDNA	*Alternaria* sp. (isolate IA2448)	AY154699	100
	Alternaria sp. (isolate IA249)	AY154698	100
	A. japonica	U05199	100
18S rDNA	*A. alternate*	U05194	100
	A. brassicicola	U05197	100
EF1a	*Alternaria* sp. (isolate CBS 174.52)	DQ677911	98
	A. alternata (isolate AFTOL-ID 1610)	DQ677927	98
cmdA*repentis*	*Pyrenophora tritici- repentis* -(isolate Pt-1C-BFP)	XM00194109	97
	A. alternata	GQ240307	97
	A. carotiincultae (isolates BMP0129)	EU141969	97
	A. carotiincultae (isolate BMP0095)	EU141972	97
act	*A. carotiincultae* (isolate BMP0132)	EU141968	97
	A. radicina (isolate BMP0047)	EU141973	91
	A. radicina (isolate BMP0062)	EU141972	91
	A. radicina (isolate BMP0079)	EU141971	91

at 50x magnifications using 7 to 14 day-old cultures. It was based on observations concerning colony growth, color, type of mycelia, size, and form arrangement of conidia. Identification key E.G.S. 00.000 was used to record conidia of this fungal isolate as described by Simmons and Roberts (1993).

RESULTS

Molecular analysis

PCR resulted in the successful amplification of ITS rDNA (541 bp) gene using primers ITS1 and ITS4, 18S DNA (527 pb) gene with primers NS1 and NS2, elongation factor 1-alpha (1182 bp) gene with primers EF1-728F and TEF1LLErev, calmodulin (1182 bp) gene with primers CAL-228 and CAL-737R and actin (240 bp) gene with primers ACT-783R and ACT-512F for isolate Mlb684. All sequences determined in this study have been submitted to GenBank. Based on DNA sequences of the 5 studied genes, sequence comparisons were performed between *Alternaria* sp. isolate Mlb684 and the closest species or isolates for which sequences were available. Comparison of the ITS rDNA gene showed that *Alternaria* sp. isolate Mlb684 was 100% identical to 3 other *Alternaria* isolates (Table 2). *Alternaria* sp. isolate MUCL 45333 is pathogen on wheat crop, in opposite, *Alternaria* sp. isolates IA2448 and IA249 that are reported infect regularly *Hylocereus undatus* fruits in Iran.

Concerning the 18S rDNA gene, *Alternaria* sp. isolate M1b684 showed 100% identity with 12 microorganisms among them 3 *Alternaria* species. *Alternaria japonica* and *A. alternata* isolated from disease plants of *Brassica rapa* ssp. *oleifera*, indeed, *Alternaria brassicicola* isolated from infected *Brassica oleracea* ssp. *capitata* in Canada. Similar to the ITS rDNA gene, the 18S rDNA gene sequence of *Alternaria* sp. isolate M1b684 showed than a 98 to 99% identity with those of 72 other *Alternaria* or unknown cultured fungi isolates making.

Comparing elongation factor 1-alpha gene sequences yielded 2 related *Alternaria* species (Table 2). *Alternaria* sp. isolate CBS 174.52 showed 98% identity along 64% of its sequence with *Alternaria* sp. isolate M1b684 while *A. alternata* isolate AFTOL-ID 1610 shared 98% identity along 61% of its sequence *Alternaria* sp. isolate M1b684.

Comparing the calmodulin gene sequence of *Alternaria* sp. isolate M1b684 to GenBank sequences yielded no *Alternaria* species sequences and the closest microorganism was the *Pyrenophora tritici-repentis* isolate Pt-1C-BFP. This fungal species shared 97% identity over 42% of its sequence. Finally when comparing the actin gene sequence of *Alternaria* sp. isolate M1b684 to GenBank sequences yielded 7 *Alternaria* species sequences ranging from 97% identity over 84% of its sequence to 91% identity along 83% of its sequence when compared. Table 2 illustrated these species. All isolates of *Alternaria carotiincultae* and *Alternaria radicina* were isolated from infected carrots. An ITS sequence (GenBank accession EU314716) for the

ITS rDNA gene of *A. eichhorniae* shared 97% of its sequence with the one of *Alternaria* sp. isolate M1b684 (GenBank accession HQ413695).

Figure 1A showed the genetic relationships between *Alternaria* sp. isolate M1b684 and the closest microorganisms for this elongation factor 1-alpha gene sequence, where it appears that it is quite distinct from the closest organisms. Genetic relationships are shown between closest calmodulin and actin gene sequences are shown respectively on Figure 1B and C. We have described phylogenetic relationships among *Alternaria* sp. isolate Mlb684 and *Alternaria* genera or isolate available in Genbank, based on sequences from five different genetic regions, ITS rDNA gene, 18S rDNA gene, EF1a gene, calmodulin gene and actin gene. ITS rDNA sequence analysis of *Alternaria* sp. isolate M1b684 presented 99% of identity to those of 93 other fungi isolates. Indeed, the 18S rDNA gene sequence of same isolate M1b684 showed also than a 98 to 99% identity with those of 72 other *Alternaria* or unknown cultured fungi isolates. In order to confirm the result obtained in phylogenies studies, a Bayesian analysis was run to this one.

Morphology

It produced an ash colony on V-8 agar and was well sporulated 2 weeks after incubation at 25°C with 16 photoperiods. Colony and conidia were easily recognizable as belonging to the *Alternaria* genus with relatively small and short conidia on branching chains. Similar descriptions were given for several species of *Alternaria* on V8 agar medium by Simmons and Roberts (1993) and Simmons (1999, 2004). Conidia of *Alternaria* sp. isolate Mlb684 are short ellipsoid to oval, tapering in the lower half into a narrow tail extension. The upper part which was materialized by a very short beak well rounded ending abruptly appears allowing the formation of new spores, thus furnishing evidence of catenulation. Primary conidiophores of *Alternaria* sp. isolate Mlb684 arise directly from hyphae at the V-8 agar surface; they can be simple or branched.

Mycelium is septate and the conidia (Figure 2A and B) are variable in size and shape, but most often short and ellipsoid to oval, tapering in the lower half. Sometimes a narrow tail extension is visible. The upper part bears a beak, but it is very short and rounded; catenulation is frequent. To our knowledge the sporulation pattern observed here, characterised by an unusually high percentage of relatively small conidia produced in non-disjunct series, and has not been observed previously in any *Alternaria* species (Figure 2C). The spores are often well formed, with septa. Most of them have a smooth wall like those of *Alternaria sesame*, *Alternaria sesamicola*, and *Alternaria simsimi* described by Simmons (2004). In cultures on potato-carrot agar, conidia E.G.S. 9-28 (-32) x 12-15 µm in size, with transverse septa and at least one longitudinal septum, were observed.

Taxonomy

Comparisons of DNA sequences, elongation factor 1-alpha gene, calmodulin gene and actin gene and morphology of isolate Mlb684 from Mali in the *Alternaria* genus revealed that the fungus from Mali represents a previously undescribed species in the genus. A new species are described as follows:

Alternaria jacinthicola Dagno & MH. Jijakli sp. nov. :Ex Colonies are described on V-8 agar. Conidiophores are abundant, heaped reaching a size range up to ca. 70 x 2-4 µm, geniculate apical growth extensions. Conidia are catenulate and produced in chain. Beak often very short rounded pouring out of the body from the conidium. Conidium bodies reach a size range of 9 to 28 (-32) x 12 to 15 µm, with 3 to 7 constricting transepta and 1 or 2 longisepta in 1 to 2 transverse sections of narrow conidia. Conidia have a smooth wall. Isolated from disease *E. crassipes* plant in Mali (Dagno et al., 2011a). Ex - Cult. Typ. BCCMTM/MUCL = MUCL 53159

Etymology- referring to the original host plant, water hyacinth. Description and observations on the ex-type culture are based on the isolate Mlb684, which was derived from infected water hyacinth plant. The material was collected in 2006 in Mali. Colony growth on V-8 and PCA is rapid, completely covering individual sectors of 90 mm diameter Petri dish within 2 weeks. Sporulation is dense on V-8, only slightly less so on PCA. Concentric rings of sporulation are evident. The conidia are obclavate (shaped like a bowling pin) and form single file chains. The spores have both 1 or 2 longitudinal and horizontal septae. Each conidium tapers into a narrow rounded protuberance. Conidium bodies reach a size range of 9 to 28 (-32) × 12 to 15 µm, with 3 to 7 constricting transepta and 1 or 2 longiseptum in 1 to 2 transverse sections of narrow conidia.

DISCUSSION

Two species of *Alternaria* genus (*A. eichhorniae* and *A. alternata*) were previously described as pathogenic fungi on water hyacinth (Aneja et al., 1989; Nag Raj and Ponnappa, 1970). This study records the discovery of an unknown species in the *Alternaria* genus. Comparisons of DNA sequences, elongation factor 1-alpha gene, calmodulin and actin of isolate Mlb684 and all *Alternaria* DNA sequences and other DNA sequences existing in GenBank, suggest that this fungus represents a new taxon, for which the name *A. jacinthicola* was provided. To date, no further examination of molecular relationships among *Alternaria* sp. isolate Mlb684 and other genera

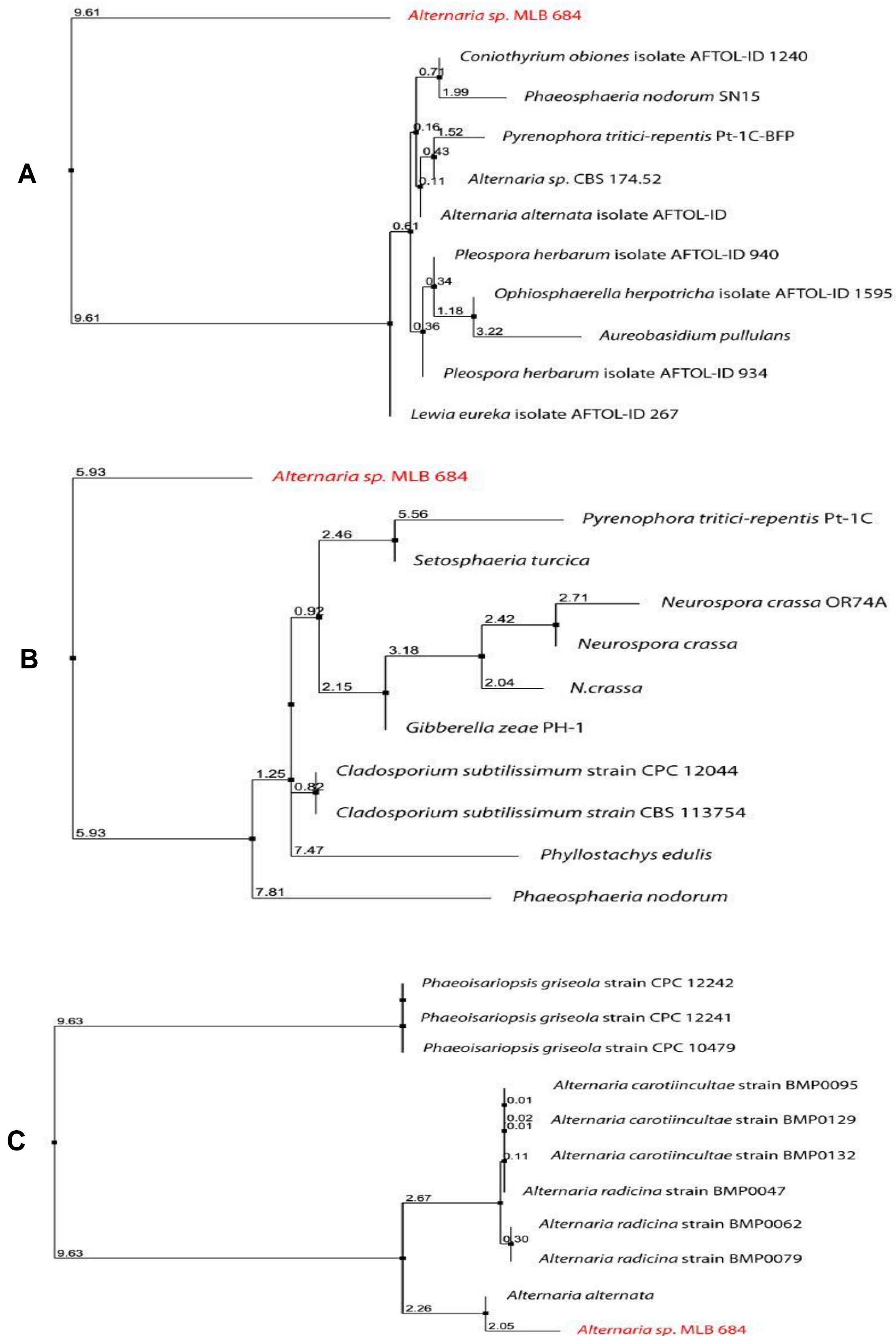

Figure 1. Molecular phylogenies of *Alternaria* sp. isolate Mlb684 based on 3 amplified and sequenced DNA regions. A) partial sequence of Elongation factor-1 alpha (EF1a) gene, B) partial sequence calmodulin gene, C) partial sequence of actin gene.

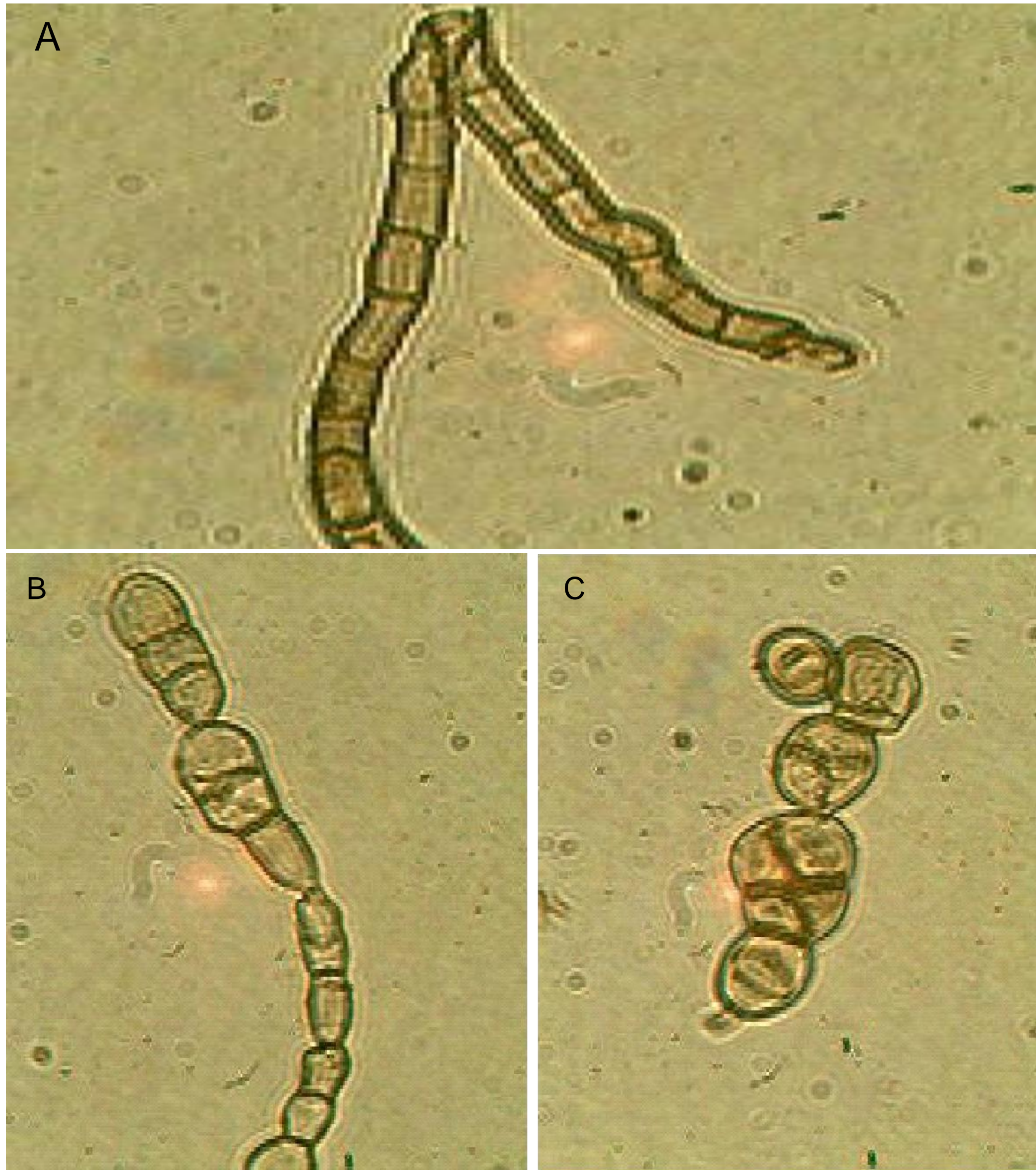

Figure 2. *Alternaria jacinthicola*. Septa mycelia (A) Conidiophore, (B) and Arrangement of conidia (C) and ex representatives of isolate Mlb684; from development of foliar disease and colony developed on V8 agar (100x magnification).

has been explored. Moreover, no analysis of this isolate Mlb684 phylogeny has ever been conducted. This work provides the first systematic examination of isolate Mlb684 as they relate to the hypothesized related taxa of *Alternaria* (Simmons, 1992). Moreover, it should be noted that some *Alternaria* species were already shown to infect water hyacinth and have been assessed as potential biocontrol agents against this weed. These are *A. eichhorniae* in Egypt (Shabana, 2005) and *A. alternata* in India (El-Morsy et al., 2006; Babu et al., 2003) which caused severe disease on the plant in greenhouse test conditions. Genetic comparisons of *Alternaria* sp. isolate Mlb684 with DNA sequences from these *Alternaria* isolates had not been possible in absence of genetic characterization of these organisms, at the exception of one sequence of *A. eichhorniae.* Additionally, isolate of *A. eichhorniae* in GenBank is not the one originally described by Shabana (1995) but by Nag Raj and Ponnappa on water hyacinth in India (Nag Raj and Ponnappa, 1970).

The objective of this phylogenetic study is to examine the relationships among *Alternaria* sp. isolate Mlb684 and the *Alternaria* genera and the closest species or isolates based on mitochondrial rDNA sequences for which sequences were available in Genbank. Results of Bayesian analysis were confirmed by the phylogenetic

data obtained in this study. Because ITS sequence of *Alternaria* sp. isolate Mlb684 was then 99% identical to those of 93 other isolates, making it not pertinent to build a molecular phylogeny. In addition, the 18S rDNA gene sequence of *Alternaria* sp. isolate Mlb684 showed also than a 98 to 99% identity with those of 72 other *Alternaria* or unknown cultured fungi isolates making so it not pertinent to build his molecular phylogeny. For morphological description, the taxon of isolate Mlb684 does not appear to be one that is identifiable with those currently recognized by E.G.S. as described in the literature (Simmons, 1999, 2004; Simmons and Roberts, 1993). Genetic results confirmed that the fungal isolate Mlb684 belonged to the genus *Alternaria* and was distinct from any *Alternaria* species and isolates which had been previously characterised.

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