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Antifungal activities of endophytic fungi isolated from plantain tissues on *Fusarium solani* a potential pathogen

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Abstract

Endophytic fungi are part of microbial community found in various types of plant tissues and display a range of symbiotic interactions with the plant host, including plantain species. They are sustainable and less harmful alternatives to chemicals in controlling pathogens. Therefore, this study was aimed to isolate the endophytic fungal communities associated with the plantain Corn 1 in order to use them for the management of root rot disease incidence. Five endophytic fungi were isolated from healthy and symptomatic parts of the plantain variety Corn1 from farms in Southern Côte d'Ivoire. The analyses of the ITS region sequences revealed that the isolates from the healthy parts LBVM Ba.0001 and LBVM Ba.0006 had 99% and 100% homology with respectively *Neosartorya laciniosa* (FR733875.1) and *Talaromyces assiutensis* (KM458833.1). The strains LBVM Ba.0002 and LBVM Ba.0003 isolated from the symptomatic parts had 99% *Fusarium solani* (KT366735.1) while LBVM Ba.0005 had 100% homology with *Nectria haematococca* (AY310442.1). Both endophytes isolates LBVM Ba.0001 and LBVM Ba.0006 were tested for their ability to inhibit the growth of *Fusarium solani* using dual culture assays. The endophytic fungi inhibited *Fusarium solani* growth significantly ($P \leq 0.05$) under *in-vitro* conditions. *Neosartorya laciniosa* showed the best biocontrol effect on *Fusarium solani* (IC50 = 0.8 g/L and IC90 = 7.6 g/L), compared to *Talaromyces assiutensis* (IC50 = 0.8 g/L and IC90 = 7.6 g/L).

Keywords: Bananas; Endophytes; Root rot disease; Biocontrol; Côte d'Ivoire

Résumé

Les champignons endophytes font partie de la communauté microbienne présente dans divers types de tissus végétaux et présentent une gamme d'interactions symbiotiques avec la plante hôte, y compris les espèces de bananier. Leur utilisation est une alternative durable et moins nocive aux produits chimiques dans le contrôle des agents pathogènes. Par conséquent, cette étude visait à isoler les communautés fongiques endophytes associées à la variété de plantain Corn1 afin de les utiliser pour la gestion de l'incidence de la pourriture des racines. Cinq souches de champignons endophytes ont été isolées de parties végétales saines et symptomatiques de champs de la variété Corn1 au Sud de la Côte d'Ivoire. Les analyses des séquences de la région ITS ont révélé que les isolats LBVM Ba.0001 et LBVM Ba.0006 présentaient respectivement une homologie de 99% avec *Neosartorya laciniosa* (FR733875.1) et avec *Talaromyces assiutensis* (KM458833.1) de la base de données GenBank. Les isolats LBVM Ba.0002 et LBVM Ba.0003 présentaient une homologie de 99% avec *Fusarium solani* (KT366735.1), tandis que l'isolat LBVM Ba.0005 présentait une homologie de 100% avec *Nectria haematococca* (AY310442.1). Les deux isolats d'endophytes LBVM Ba.0001 et LBVM Ba.0006 ont été testés pour déterminer leur capacité à inhiber la croissance de *Fusarium solani* à l'aide de tests de double culture. Les champignons endophytes ont fortement inhibé la croissance de Fusarium solani ($P \leq 0,05$) dans des conditions *in vitro*. *Neosartorya laciniosa* a montré le meilleur effet de biocontrôle sur *Fusarium solani* (IC50 = 0,8 g / L et IC90 = 7,6 g / L) par rapport à *Talaromyces assiutensis* (IC50 = 0,8 g / L et IC90 = 7,6 g / L).

Mots-clés: plantain; endophytes; Maladie de la pourriture des racines; Biocontrôle; Côte d'Ivoire

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Introduction

Endophytic fungi are polyphyletic and belong mainly to the family Ascomycetes and their anamorphs (Aly et al., 2011). Their presence has been noted in the tissues of almost all plants including algae (Hawas et al., 2012), mosses and lichens (U'Ren et al., 2010), several angiosperms and gymnosperms such as tropical palmtrees, woody species (Arnold et al., 2003), various annual herbaceous plants, and many deciduous and evergreen plants, in all climatic regions around the world (Petrini et al., 1992). Fungi described as endophytes have a period during which their growth and colonization stop momentarily, resuming after a change in physical conditions or maturation within the host (Zuccaro et al., 2014). This type of growth is a definite characteristic of endophytes, although they are sometimes considered, in the end, as commensals, latent pathogens or protective mutualists. Recent studies have shown that endophytic fungi are able to protect their hosts from disease and limit the damage caused by pathogenic microorganisms (Omomowo and Babalola, 2019). Indeed, endophytic fungi can produce secondary metabolites having antifungal and antibacterial properties (Toghueo and Boyom, 2019). These compounds can significantly inhibit the growth of other microorganisms including plant pathogens. These endophytes might be a more sustainable alternative to chemicals that threaten human health and the environment. Bananas including plantain, are the most important crop in the southern region of Côte d'Ivoire (Faostat, 2019). However banana productivity is thwarted by different diseases caused by fungi pathogens. Fusarium species are among the most aggressive telluric fungi responsible for wilting and root rot in many plant species, including plantain (Randy, 2006). Panama disease, which is also known as fusarium wilt due to *Fusarium oxysporum* f. sp. cubense, Foc, is regarded as one of the most destructive and widespread diseases of banana production worlwide (ref). Moreover, strains of *Fusarium solani* and their telomorphs *Nectria hematococca* can cause plantain root rot (Meddah et al. 2011). In Côte d'Ivoire, a clear cut characterizasation of the pathogen responsible of banana fusarium diseases has not been done. However in 2006, characteristic symptoms of Fusarium wilt were observed in several Cavendish (AAA) and Corn1 plantations in South Côte d'Ivoire. Indeed Fusarium isolates from banana plantations on the outskirts of Abidjan were shown to have macroscopic and microscopic characteristics similar to those of *F. oxysporum* f. sp. Cubense, and were able to induce characteristic symptoms of Fusarium wilt (Kra et al., 2011). Yet the pathogen was not clearly identified while the average incidence of Fusarium wilt in Abidjan and Anyama was 54.07% and 63.45%, respectively, on the cultivars Grande Naine and Corn1. Moreover characteritics root rot diseases were also observed. It was then urgent to identify and characterize the pathogenic fungi involved in banana these diseases in order to develop a cost-effective measure for an efficient control in Southern Côte d'Ivoire. The use of endophytic fungi were shown to be efficient on pathogens of different plants (Talapatra et al. 2017). They could be used as an efficient alternative for the biological control of plantain root rot disease. The present study aimed at isolating both the pathogenic fungus involved in the root rot disease in Southern Côte d'Ivoire and endophytic fungi colonizing different healthy plant parts of the plantain Corn1 variety, and evaluating the effect of these fungal endophytes on the root rot pathogen under controlled conditions.

Materials and Methods

Tissue sampling

The plantain Corn 1 variety tissues were sampled from 04 farm plots in the Southern Region of Côte d'Ivoire. The incidence of the fungal root-rot symptoms in the plots was above 80%. The aim of the sampling was to collect both plantain healthy and symptomatic tissues in order to isolate potential endophytes and the pathogenic fungus. The samples consisted of plantain roots and pseudostem sections. Per plot, 03 plants were selected randomly.

Per plant, 03 root tips and 03 vascular bundle sections of the pseudostem were collected.
The tissues were stored under sterile conditions and sent to the Laboratory of Plant and Microbial Biotechnology (LBVM) of INP-HB, Ivory Coast.

Culture and purification of fungal strains
Isolation of fungi from plantain organs was carried out according to the method of Davet & Roux (1997) on solidified PDA medium in a Petri dish. Monospore cultures from the fungi colonies were performed for 02 weeks using the method of Ho & Ko (1997). Endophyte isolates were grouped by morphotype (Botton et al., 1990 ; Champion, 1997).

PCR amplification of the ITS region
The spores and mycelium of each strain were collected and ground in a mortar. The DNA was then extracted using PowerSoil Power Extraction Kit. The extracted DNA was stored at -20°C. Primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGCGC-3') (White et al. 1990) were used for PCR amplification of the fungal ITS region. For the PCR amplification, the reaction medium was composed of 25 µl of PCR master-mix and 2 µl of DNA sample. The PCR master-mix consisted of 2.5 µL of 10 × buffer (MP Biomedical), 2 µL of dNTP (2.5 mM), 2.5 µL of ITS1 (2 µM), 2.5 µL of ITS4 (2.5 µM), 0.2 µL Taq polymerase (15U / µl) and 13.5 µL of ultra-pure sterile water. The PCR amplification was carried out in a thermocycler using the following program: initial denaturation for 5 min at 95 ° C, followed by 30 cycles of 94 ° C (30 sec), 58 ° C (30 sec), 72 ° C (1 min), and final elongation at 72 ° C for 7 min.
The PCR amplicons were resolved using electrophoresis in 1.2% agarose gel and TAE buffer (40mM Tris pH 7.8, 20mM acetic acid, 2mM EDTA). Amplification bands were stained with ethidium bromide and revealed under UV light.

Sequencing and phylogenetic analyses
Sequencing of the amplicons was performed by GATC Biotech (Konstanz, Germany). The sequence comparisons were done by BLAST in GenBank databases (http://www.ncbi.nlm.nih.gov/genbank). Phylogenetic analysis was carried out using the Neighbor-joining method (Saitou and Nei, 1987) with MEGA 6 software (Tamura et al., 2013).

Dual culture assay
A dual culture assay was carried out to assess the antagonism between the identified root rot pathogen and each isolated endophyte, according to the method described by Blumenstein (2015). A 5 mm diameter disc from growing edge of a 7-day old the identified root rot pathogen and each endophytic fungal culture maintained on PDA were placed at the two opposite ends of a PDA plate. There were 03 replicates for each endophyte tested. After 07 days of culture, the colonization of the endophyte was measured by the following formula:

$$C(\%)=\frac{DT}{DE}$$

- DT is the growth distance of the endophyte on the axis between the 02 antagonists discs
- DE is the distance the antagonists discs.

Effect of endophytes ethanolic extract on the identified root rot pathogen
Each endophyte was cultivated in a flask containing a PG liquid medium for 07 days, then 250 mL of ethanol was added to each flask. The mixture was left overnight to stop cell growth. The culture media and mycelia were then milled in a blender for 10 minutes in order to kill cells and filtered through Wattman paper. The mycelium was discarded, and the culture filtrate transferred to a separatory funnel. The ethanoic and aqueous phases were separated, and the aqueous phase underwent two more extractions with 300 mL of ethanol each. All the extracts obtained were dried under reduced pressure at 40 ° C (Indira et al., 2015).
After evaporation of the solvent, the dry extract was incorporated in Czapeck medium in the following concentrations: 0.5; 1; 5; 10; 25 and 50 g / L. In each case, each was inoculated with an explant of the identified root rot pathogen. The control consisted of medium Czapek without extract (Essalmani & Lahlou, 2002). There were 03 replicates for each endophyte and the Percentage of growth inhibition (I%) was estimated:
PW: weight of mycelium in the absence of endophyte extract
BW: weight of the mycelium in the presence of endophytes ethanolic extract
IC50 and IC90 values were extrapolated from two graphical approaches.

Results

Molecular identification of endophytic fungi isolated from plantain

Following purification of the mycelial cultures, 05 fungal strains were obtained of which two from healthy tissues and three from symptomatic tissues : LBVMBa.0001, LBVMBa.0002, LBVMBa.0003, LBVMBa.0005, and LBVM Ba.0006. (Figure 1).

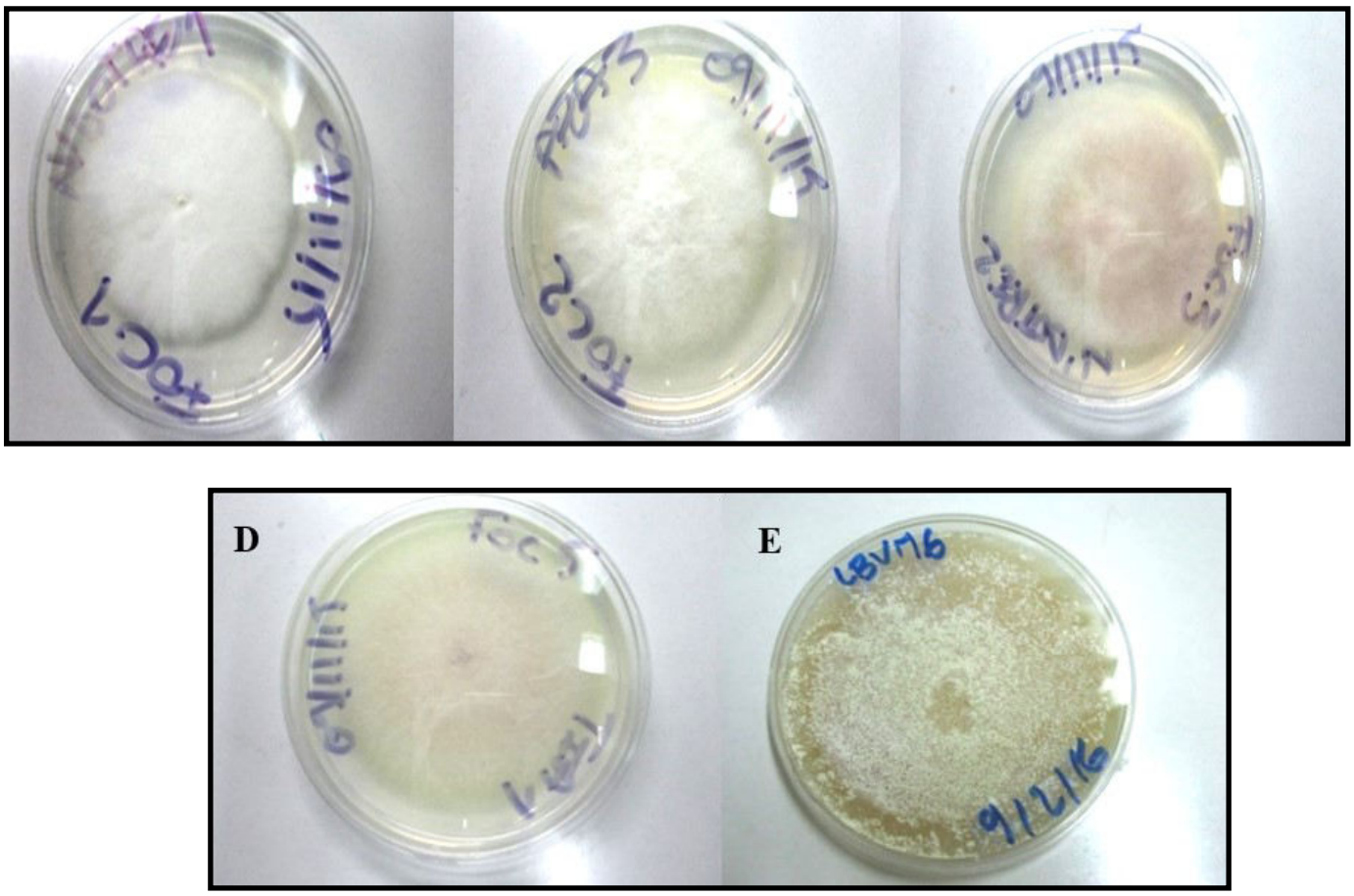

Figure 1. Fungal isolates after 07 days culture in PDA medium. A : LBVM Ba.0001; B : LBVM Ba.0002; C : LBVM Ba.0003; D : LBVM Ba.0005; and E: LBVM Ba.0006

After PCR amplification of the ITS region, an expected 550 bp fragment was obtained. BLAST analyses showed that the ITS sequence amplified from the strain LBVM Ba.0001 had a 99% homology with that of the strain Neosartorya laciniosa CCF: 1734 (SEQ ID: FR733875.1) from the GenBank database. Phylogenetic analysis confirmed that strain LBVM Ba.0001 was identified as Neosartorya laciniosa (Figure 2). The BLAST also showed a 99% homology between the ITS region sequence of the strain LBVM Ba.0006 and the ITS region sequence of the strain *Talaromyces assiutensis* NFML_CH56_421 (Sequence ID: KM458833.1) from the GenBank database. This was also confirmed by phylogenetic identification (Figure 3).

Fusarium solani was isolated from the symptomatic tissues

Blast analyses of the ITS sequences obtained from strains LBVM Ba.0002 and LBVM Ba.0003 obtained from symptomatic tissues showed 99% homology with Fusarium solani JM6201508003 (SEQ ID: KT366735.1) from databases while the strain LBVM Ba.0005 had 100% homology with the strain Nectria haematococca (SEQ ID: AY310442.1). Phylogenetic analyses confirmed the identification of LBVM Ba.0002 and LBVM Ba.0003 as *Fusarium solani* and strain LBVM Ba.0005 as *Nectria haematococca* its telomorph (Figure 4).

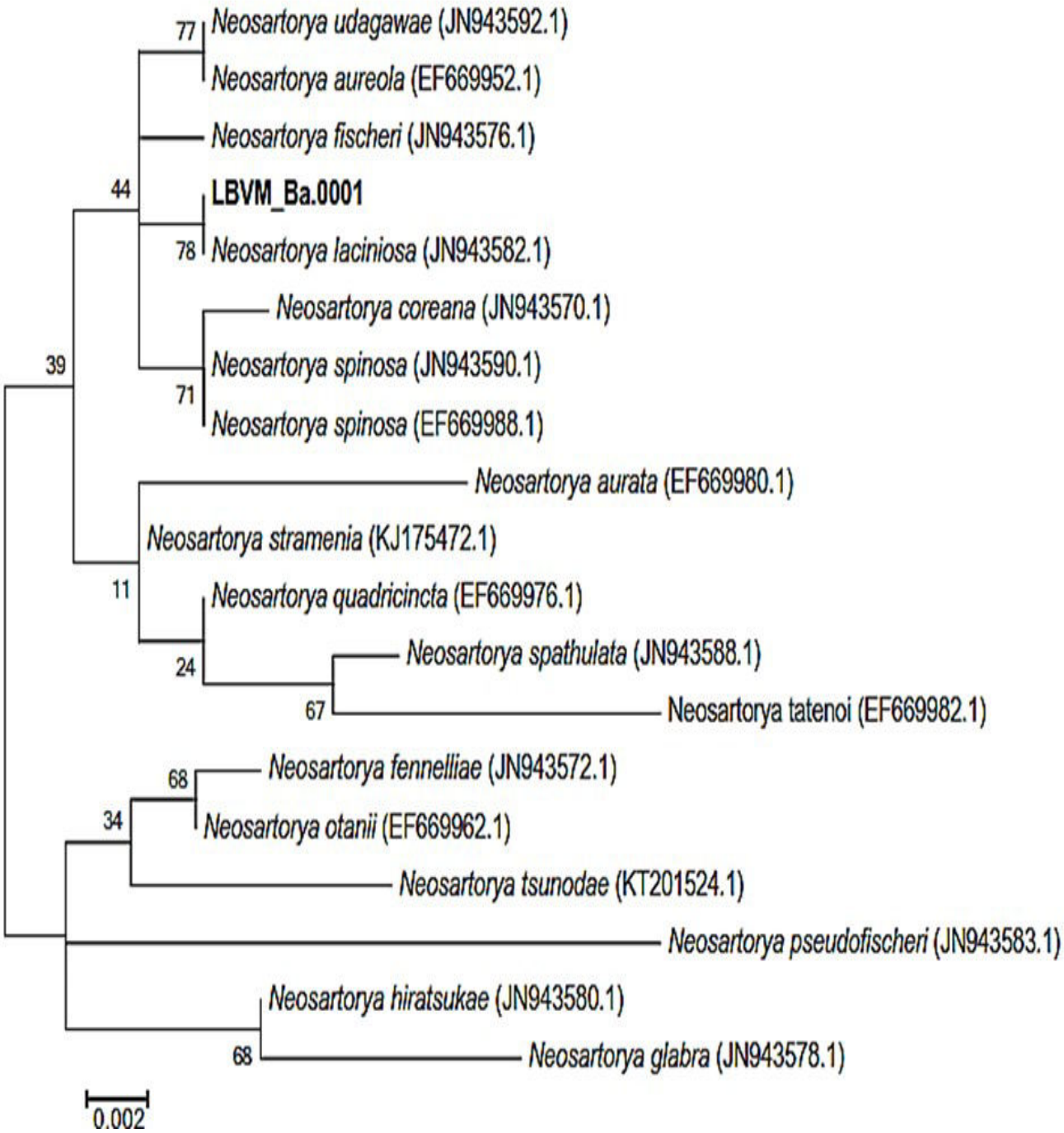

Figure 2 Phylogenetic identification LBVM Ba.0001 isolated from healthy tissues. ITS gene sequences from fungi species: *Neosartorya fischeri* (JN943576.1), *Neosartorya coreana* (JN943570.1), *Neosartorya spinosa* (JN943592.1), *Neosartorya aureola* (EF669952.1), *Neosartorya laciniosa* (JN943582.1), *Neosartorya spinosa* (EF669988.1), *Neosartorya stramenia* (KJ175472.1), *Neosartorya aurata* (EF669980.1), *Neosartorya tsunodae* (KT201524.1), *Neosartorya quadricincta* (EF669976.1), *Neosartorya fenneliae* (JN943572.1), *Neosartorya otanii* (EF669962.1), *Neosartorya pseudofischeri* (JN943583.1), *Neosartorya spathulate* (JN943588.1), *Neosartorya tatenoi* (EF669982.1), *Neosartorya hiratsukae* (JN943580.1), and *Neosartorya glabra* (JN943578.1) were used for comparison. The tree was constructed by the neighbor-joining method using Mega version 6

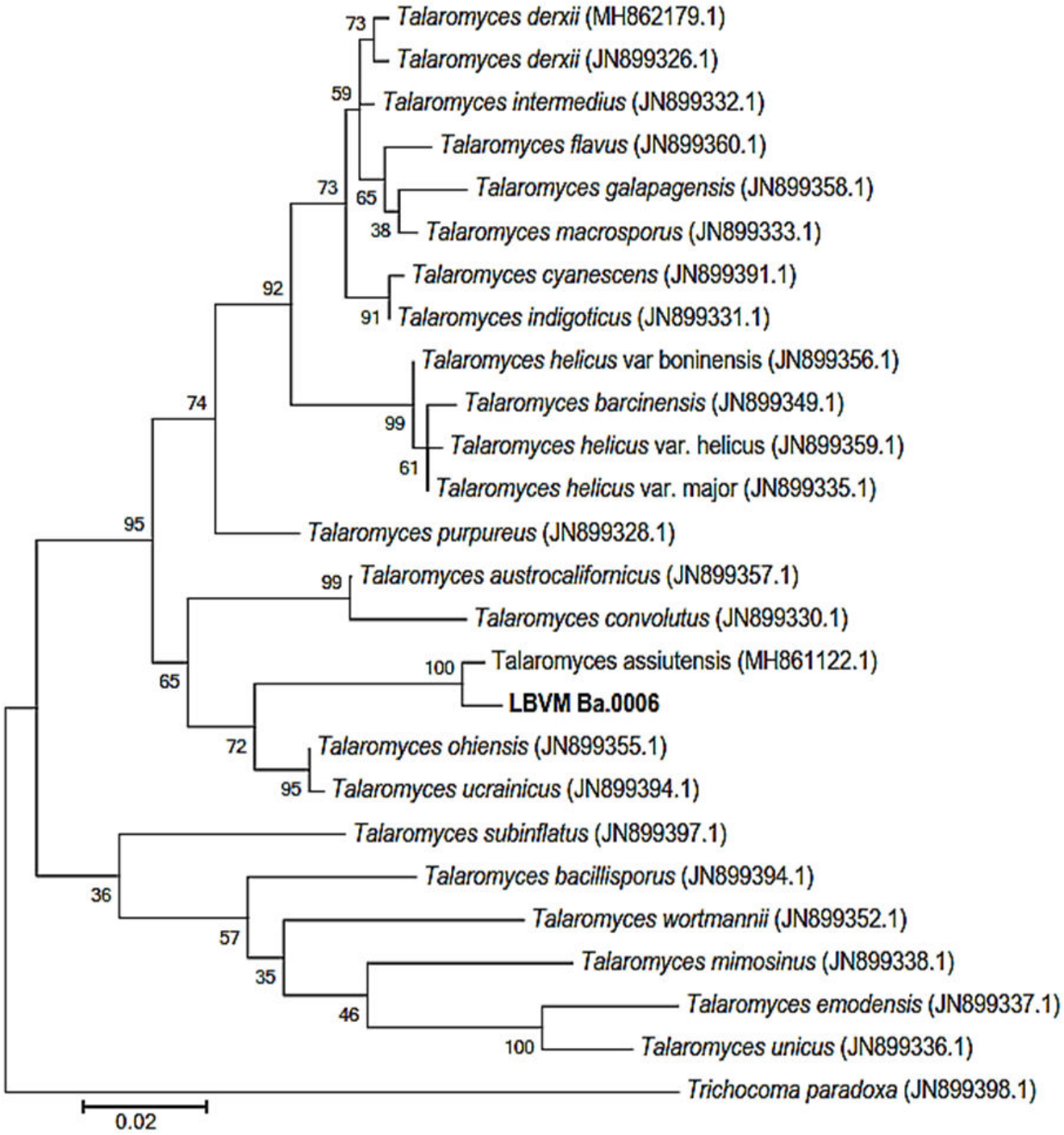

Figure 3. Phylogenetic identification LBVM Ba.0006 isolated from healthy tissues. ITS gene sequences from fungi species : *Talaromyces derxii* (MH862179.1), *Talaromyces derxii* (JN899326.1), *Talaromyces intermedius* (JN899332.1), *Talaromyces galapagensis* (JN899358.1), *Talaromyces flavus* (JN899360.1), *Talaromyces macrosporus* (JN899333.1), *Talaromyces cyanescens* (JN899391.1), *Talaromyces indigoticus* (JN899331.1), *Talaromyces helicus var. boninensis* (JN899356.1), *Talaromyces barcinensis* (JN899349.1), *Talaromyces helicus var. helicus* (JN899359.1), *Talaromyces helicus var. major* (JN899335.1), *Talaromyces purpureus* (JN899328.1), *Talaromyces austrocalifornicus* (JN899357.1), *Talaromyces convolutus* (JN899330.1), *Talaromyces assiutensis* (MH861122.1), *Talaromyces ohiensis* (JN899355.1), *Talaromyces ucrainicus* (JN899394.1), *Talaromyces bacillisporus* (JN899394.1), *Talaromyces wortmannii* (JN899352.1), *Talaromyces mimosinus* (JN899338.1), *Talaromyces emodensis* (JN899337.1), *Talaromyces unicus* (JN899336.1), *Talaromyces subinflatus* (JN899397.1), and *Trichocoma paradoxa* (JN899398.1) were used for comparison. The tree was constructed by the neighbor-joining method using Mega version 6.

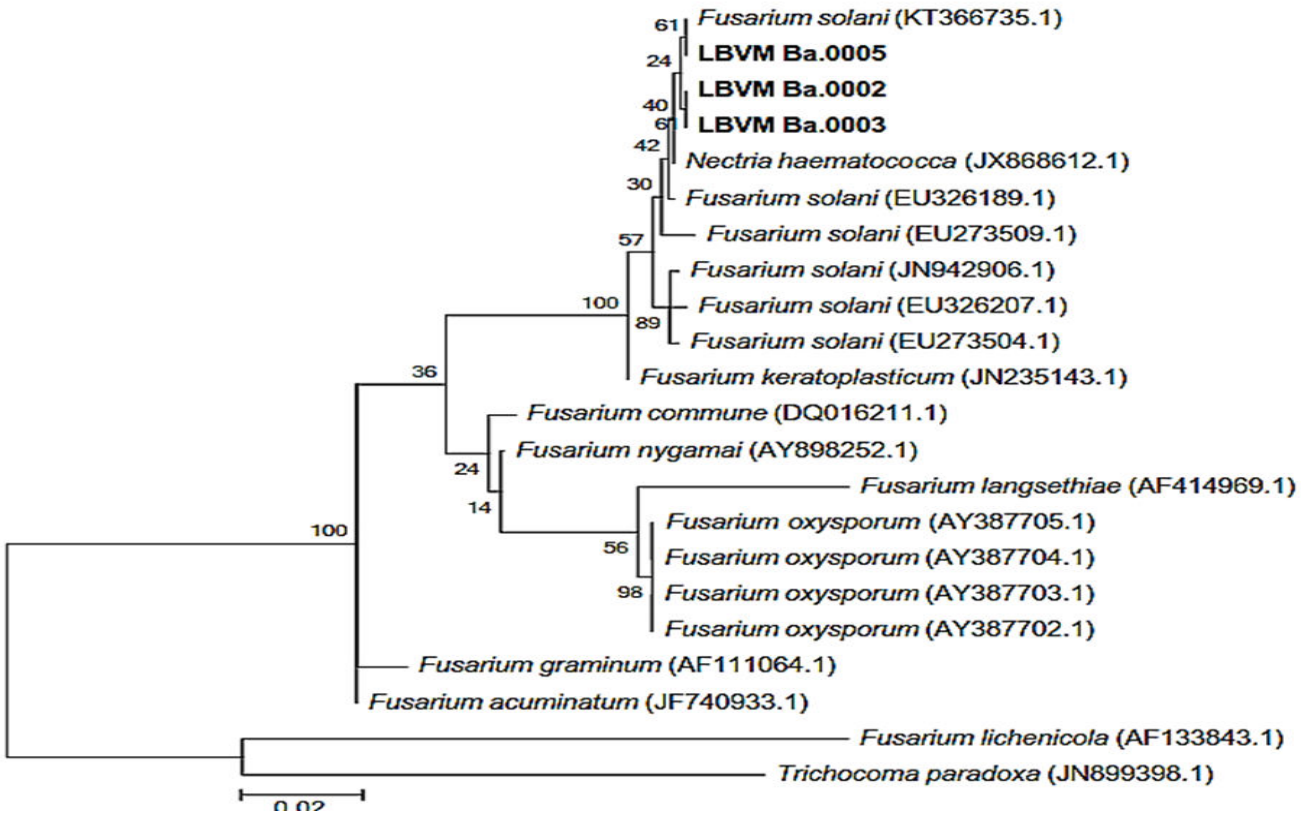

Figure 4. Phylogenetic identification LBVM Ba.0002, LBVM Ba.0003 and LBVM Ba.0005 isolated from symptomatic banana tissues. ITS gene sequences from *Fusarium solani* (KT366735.1), *Nectria haematococa* (JX868612.1), *F. solani* (EU326189.1), *F. solani* (EU273509.1), *F. solani* (JN942906.1), *F. solani* (EU326207.1), *F. solani* (EU273504.1), *F. keratoplasticum* (JN235143.1), *F. nygamai* (AY898252.1), *F. commune* (DQ016211.1), *F. acuminatum* (JF740933.1), *F. graminum* (AF111064.1), *F. langsethiae* (AF414969.1), *F. oxysporum* (AY387705.1), *F. oxysporum* (AY387704.1), *F. oxysporum* (AY387703.1), *F. oxysporum* (AY387702.1), *F. lichericola* (AF133843.1) and an outgroup *Trichpcoma paradoxa* (gi374093520) were used for comparison. The tree was constructed by the neighbor-joining method using Mega version 6

Antifungal activities of the isolated endophytic fungi on *Fusarium solani*

The interaction between the endophytes and F. solani during the dual culture assays was evaluated. In all plates, there was an "inhibition zone" where *F. solani* strain was unable to extend its mycelium. *Neosartorya* laciniosa strain covered on average 75% of the Pretri dish whereas Talaromyces assiutensis colonized on average 60.75% of the plate in the presence of *Fusarium solani* (Table 1) and (Figure 5).

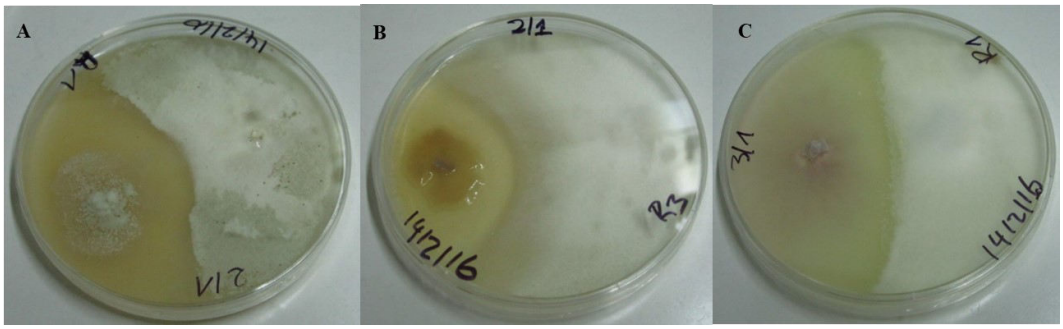

Figure 5. Endophytes and *Fusarium solani* in dual culture showing antagonism (07 days old cultures). Colony on the left hand side represents *Fusarium solani* and colonies on the right hand side represent endophytes: A)*Talaromyces assiutensis*; B & C) *Neosartorya laciniosa*

Table 1. Dual culture between the endophytes and *Fusarium solani*

	Fusarium solani
Neosartorya laciniosa	75 ± 2,75% a
Talaromyces assiutensis	60,75 ± 1,75% b
	Kruskall-Wallis ranks
Neosartorya laciniosa	15
Talaromyces assiutensis	6
	p = 0,049

Effect of endophytes ethanolic extract on *Fusarium solani*

After 07 days of culture, the half maximal inhibitory concentration (IC50) of *Fusarium solani* culture was 0.8 g / L and IC90 of this culture by the strain *Neosartarya laciniosa* was 7.6 g / L. As for the strain *Talaromyces assiutensis*, the inhibition concentrations of *Fusarium solani* were respectively: IC50 = 5.6 g / L and IC90 = 40.5 g / L (Figure 6).

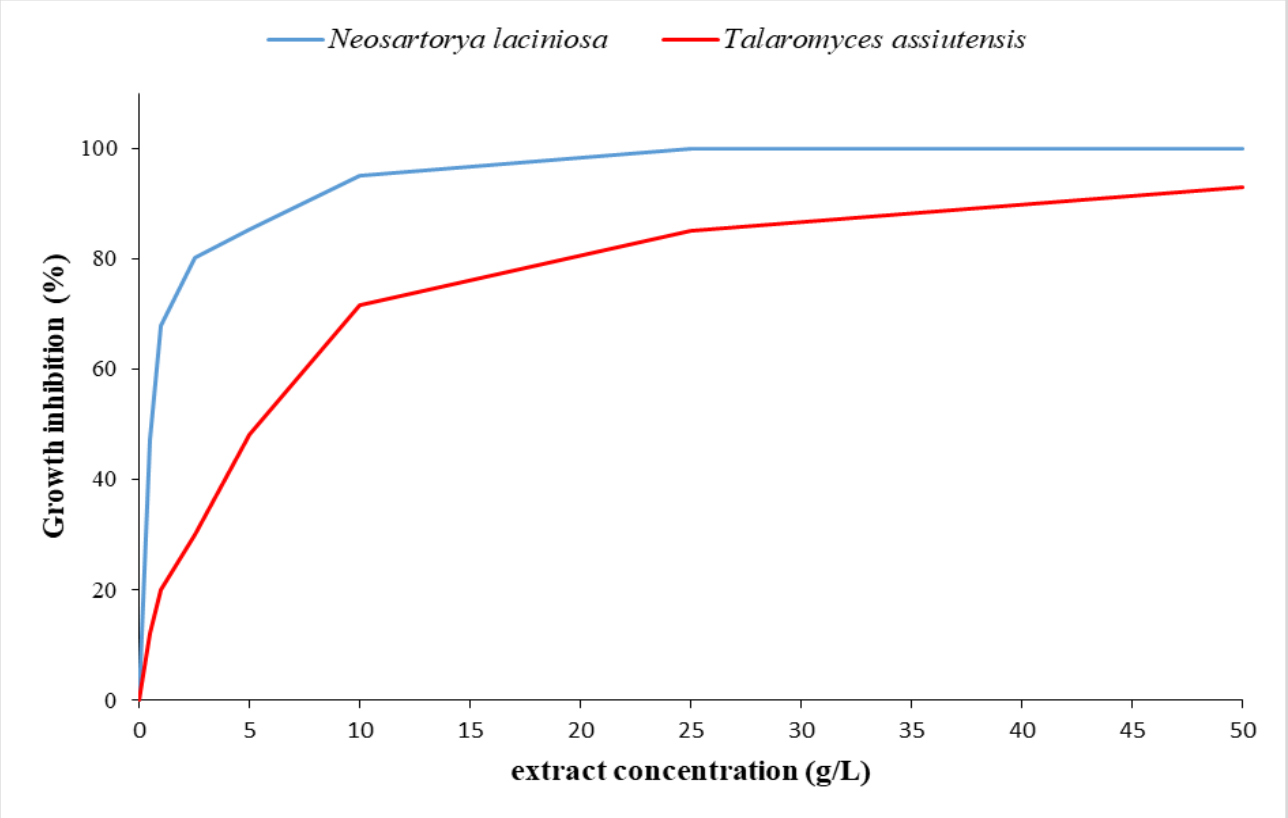

Figure 6. Curve of *Fusarium solani* growth as affected by *Neosartorya laciniosa* and *Talaromyces assiutensis* ethanolic extracts after 07 days of culture.

Discussion

The objective of this study was to identify the pathogenic fungus that caused suspected banana root rot disease observed on the variety Corn1 in Southern Côte d'Ivoire. Five fungal strains were then isolated from banana symptomatic tissues. The ITS sequence analyses allowed the identification of two strains as *Fusarium solani* and one strain as its telomorph that might be responsible of root rot symptoms in banana plantations in Southern Côte d'Ivoire. Despite the fact that this fungus has been identified elsewhere as responsible for plantain root rot and pseudostem truncation (David, 1997; Meddah et al., 2011), it has never been detected in Côte d'Ivoire as plantain root rot pathogen. Yet *Fusarium solani* agressivity has to be confirmed in both controlled and field conditions. In order to develop biocontrol strategy for this *F. solani* strain, endophytic fungi were isolated from banana healthy tissues. Only two fungal species including *Neosartarya laciniosa* and *Talaromyces assiutensis* were isolated. Recently not only a higher number of endophytes was isolated from banana (Zakaria and Aziz, 2018) but also *Neosartarya laciniosa* and *Talaromyces assiutensis* were not evidenced. The importance of endophytic strains belonging to the genera Neosartorya and Talaromyces were previously shown to have an effective biocontrol effect on different pathogens according to several studies (Eamvijarn et al., 2012; Boonsanga et al., 2014; Kumla et al., 2014; Dethoup et al., 2015). It is known that strains belonging to these genera produce secondary metabolites with interesting biological activities (Dethoup et al., 2007; Eamvijarn et al., 2012; 2013). The dual culture assays showed that *Neosartorya laciniosa* and *Talaromyces assiutensis* colonized a large proportion of the culture medium against *Fusarium solani*. Inhibitory activities against *F. solani* suggested that *Neosartorya laciniosa* and *Talaromyces assiutensis* might release chemical compounds that affected the pathogenic strain's growth. Both endophytic fungi did not control *F. solani* inhibition the same way. Indeed *Neosartorya laciniosa* was more effective (IC 50 = 0.8 g / L; IC 90 = 7.6 g / L) than *Talaromyces assiutensis* (IC 50 = 5.6 g / L; CI90 = 40.5 g / L) on the inhibition of *Fusarium solani* growth. This would be due to greater sensitivity of the strain of *Fusarium solani* to the secondary metabolites produced by *Neosartorya laciniosa*. However, an *in vitro* study conducted by Dethoup et al. (2015) showed a higher efficiency of the strain *Talaromyces trachyspermus* compared to the strain *Neosartorya pseudofischeri* on a *Fusarium strain.*

Conclusion

This is the first time the pathogen responsible of banana root rot diseases in Côte d'Ivoire was identified. *Neosartorya laciniosa* and *Talaromyces assiutensis* ethanolic extracts had the potential to be used to prevent Banana seedlings from showing fungal root rot disease symptoms. This offer the possibility to use banana endophytic fungi as efficient alternative for the biological control root rot disease in Côte d'Ivoire.

Acknowledgments

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