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Phylogenetic Characterisation of Antimycotoxigenic Fungal and Bacterial Endophytes from Maize Roots

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Abstract

Endophytes are interesting microorganisms with ability to produce important health and industrial relevant metabolites. This study characterised the phylogenic relationship of bacterial and fungal endophytes, and determined the antimicrobial potential of the endophytes against mycotoxigenic fungi of stored maize. Maize roots were collected from which endophytes were isolated. The isolates' DNA was amplified and sequencing carried out to identify them. The phylogenetic relationship of the isolates was constructed from the obtained sequences. The antifungal activity of the isolates was determined against mycotoxigenic fungi obtained from maize seeds. Fourteen isolates (six bacteria and eight fungal strains) were identified as endophytes. *Trichoderma harzianum* had activity against *Penicillium verrucosum, Aspergillus carbonarius, A. parasiticus, A. ochraceus, A. flavus* and *A. niger* with inhibition ranging from 78.6 to 90.6 %, while *Burkholderia cepacia* had percentage inhibition of 60.9 and 63.2 % against *A. ochraceus* and *A. niger*, respectively as the most active antifungal bacterial endophyte. The phylogenetic revealed that *Burkholderia cepecia* and *Novosphingobium* sp. are not closely related to the other bacterial isolates.

Keywords: Endophytes, Maize roots, Antifungal activity, Mycotoxigenic fungi

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Introduction

Endophytes are associated with plant tissues, forming communities maintained with secondary metabolites that mitigate stress, protect against infections and confer improved immune function on the plant host [1]. These microorganisms (bacteria and fungi) establish interactive mutual and symbiotic relationships that do not result in disease symptoms or infection in the plant host [2]. Microorganisms are introduced into plants through the root system, an interphase (rhizosphere) between the host plant and the soil environment. The rhizosphere environment is characterised by a large concentration of metabolites from the environment, exudates released by plant roots, an array of microorganisms and functional relationships in such environments.

Endophytes are categorised into the Clavicipitaceous group, which establishes relationships with a narrow range of plant hosts and is transmitted vertically through ovules to progeny plants through seeds, and the non-Clavicipitaceous endophytic group, which has a broad host range. Non-Clavicipitaceae fungi access plants through roots, shoots and rhizomes, can remain dormant in the host and can be transmitted vertically or horizontally to subsequent progeny. The number and concentration of secondary metabolites produced are factors that are induced by the endophytic microbial community of silent gene clusters that regulate metabolite production in plants and stimulate the plant immune system [3]. Clustering of genes responsible for the synthesis of metabolites results in two secondary phenotypic products: (1) gene clusters working together to yield complex secondary metabolites, and (2) the modulation of different pathways for secondary metabolite production [1, 4].

The problems of the emergence of new diseases and re-emergence of diseases, which are propelled by increasing population size, climate change and resistance to antimicrobial agents, have increased the demand for available antimicrobial agents, thus creating a gap in the supply and availability of environmental friendly and efficient antimicrobial agents. Endophytes have been explored to bridge this gap, as they are known producers of novel metabolites. They occupy unique ecological niches in the environment. The quest for newer and more potent drugs to combat these challenges is increasing.

The interaction between the host plant and the endophyte results in the production of metabolites that suppress the effects of biotic and abiotic stressors on the plant and promote development and defensive responses to external aggressors. The mass production of secondary metabolites for large-scale use is still in its infancy because of poor understanding of the metabolic pathways involved, the effects of plant-endophyte interactions on metabolite production and what induces the production of a particular metabolite [2, 5].

Materials and Methods

Isolation of endophytes from plant roots

Maize root samples were collected from Lafia, Nigeria, following the procedure described by Orole *et al.* [6]. Fifteen roots (50 g each) among the collected samples were cleaned, sterilised with 0.8 % hypochlorite solution and rinsed three times with distilled water. Sterilized roots (1 g) were inoculated on nutrient agar and potato dextrose agar for bacterial and fungal isolates, respectively, at 27 °C for 48-78 h.

16S rRNA/ITS sequencing of bacterial and fungal isolates

Bacterial and fungal DNA were extracted, purified and used for their respective PCR mixtures. Amplification of the fungal PCR mixture was carried out with ITS1 (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'- TCCTCCGCTTATTGATATGC-3') primers, while bacterial DNA was amplified using F27 (5'-AGAGTTTGATCMCTGGCTCAG-3') and R1492 (5'-TACGGYTACCTTGTTACGACTT -3') primers according to the methods of Orole *et al.* [6]. Sequencing was carried out, and the sequences were edited and aligned using Geneious bioinformatics software (version 8.1). The obtained sequences were searched for homology using the Basic Local Alignment Search Tool (BLAST) software algorithm at NCBI GenBank (http://www.ncbi.nlm.nih.gov.blast). The obtained data were deposited at NCBI. The phylogenetic relationships of the isolates were constructed from the obtained DNA sequences via MEGA 11.

Determination of the antifungal activity of the root endophytes

The antifungal activity of the endophytes was tested on mycotoxins produced by fungi (*Penicillium verrucosum*, *Aspergillus carbonarius*, *A. niger*, *A. flavus*, *A. parasiticus* and *A. ochraceus*) isolated from maize grains sourced from Nigerian markets (*Supplementary file 1*). Fungal and bacterial endophytes were removed from 3-day-old cultures (5 mm each), inoculated against the toxigenic fungal strains at opposite ends, and incubated for 5 days at 28 °C after which zones of inhibition were measured. The control cultures were made of 5 mm of toxigenic fungi grown on PDA.

Phylogenetic analysis of bacterial and fungal endophytic strains

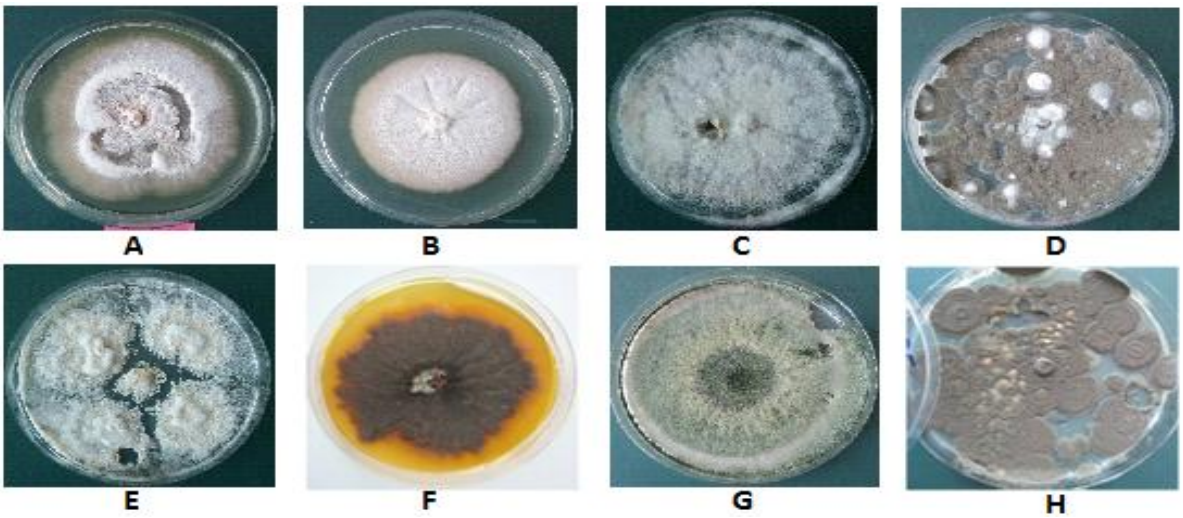
The evolutionary history was inferred using the neighbor-joining method. The bootstrap consensus tree was inferred from 1000 replicates, and the evolutionary distances were computed using the maximum composite likelihood method involving 7 bacterial and 6 fungal nucleotide sequences. Evolutionary analyses were conducted in MEGA11 [7].

Results and Discussion

A total of fourteen endophytes (Table 1) were isolated (eight from fungi and six from bacteria). The fungal ITS sequences have been deposited at the GenBank and allotted accession numbers (https://submit.ncbi.nlm.nih.gov/subs/?search=SUB6216550) as previously reported by Orole *et al.* [6]. The bacterial DNA sequences were used to assign species names after performing a BLASTn search on the NCBI database. The colonial form of the fungal endophytes is presented in Fig. 1.

Table 1: Fungal and bacterial endophytes from maize roots

Name of Endophyte	Strain name	NCBI Accession No
Fungal Strains		
Talaromyces verruculosus	LF112	MN393465
Dichotomopilus erectus	hk2619	MN393466
Clonostachys rosea	RT075	MN393467
Talaromyces sp.	JNL121	MN393468
Trichoderma harzianum	kk073	MN393469
Acremonium sp.	DAM107	MN393470
Fungi sp	TIM053	MN393471
Trichoderma sp	TOA55	MN393472
Bacterial Strains		
Micrococcus luteus	-	-
Bacillus sp.	-	-
Burkholderia cepecia	-	-
Bacillus licheniformis	-	-
Bacillus licheniformis	-	-
Novosphingobium sp	-	-

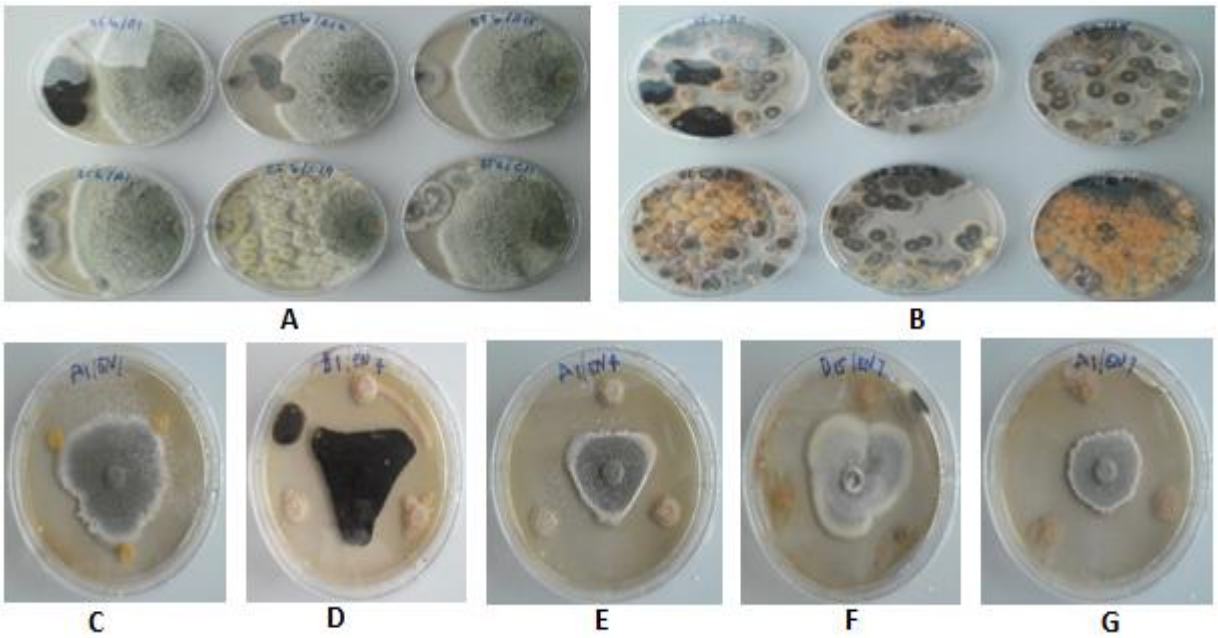


[(A = Fungal strain TIM053; B = *Acremonium* sp. strain DAM107; C = *Trichoderma* sp. strain TOA55; D = *Talaromyces* sp. strain JNL121; E = *Clonostachys rosea* strain RT075; F = *Dichotomopilus erectus* strain hk2619; G = *Trichoderma harzianum* strain kk073; H = *Talaromyces verruculosus* strain LF112)]

Figure 1: Colonial structures of endophytic fungal species from maize roots

Table 2: Inhibitory activity of endophytes against mycotoxin-producing fungi

Toxigenic fungi	Control	*D. erectus*	*T. harzianum*	*T. verruculosus*	*B. cepecia*	*Novosphingobium* sp
		Radial growth in mm (% Inhibition)				
P. verrucosum	14.0	6.3±0.6 (55.0)	3.0±0.6 (78.6)	7.3±0.8 (47.9)	6.7±0.6 (52.1)	11.0±0.5 (21.4)
A. carbonarius	19.0	7.7±1.5 (59.5)	3.0±1.6 (84.2)	6.7±1.3 (64.7)	12.3±1.2 (35.3)	10.3±5.3 (45.8)
A. parasiticus	14.0	9.0±1.7 (35.7)	4.0 (71.4)	5.0±1.4 (64.3)	7.0±1.0 (50.0)	9.7±5.6 (30.7)
A. ochraceus	23.0	10.0 (56.5)	4.0 (82.6)	14.0±0.1 (39.1)	9.0±1.0 (60.9)	19.0±0.1 (17.4)
A. flavus	32.1	10.0±2.0 (68.8)	3.0 (90.6)	23.7±2.1 (25.9)	26.0±2.5 (18.8)	22.0±2.2 (31.3)
Aspergillus sp.	15.0	8.3±1.5 (44.7)	3.0 (80.0)	9.3±0.5 (38.0)	5.7±0.6 (62.0)	12.0±1.6 (20.0)
A. niger	38.0	12.1±3.3 (68.2)	8.1±3.3 (78.7)	15.3±2.5 (59.7)	14.0±2.0 (63.2)	12.0±2.8 (68.4)



[A = *Trichoderma harzianum* strain kk073 (EF6) against B1 (*A. niger*), C12 (*A. parasiticus*), D15 (*A. versicolor*), A1 (*A. versicolor*), *and* C19 (*A. fumigatus*); B = *Dichotomopilus erectus* strain hk2619 (EF2) against B1 (*A. niger*), C12 (*A. parasiticus*), D15 (*A. versicolor*), A1 (*A. versicolor*), *and* C19 (*A. fumigatus*); C = Endophyte *Micrococcus luteus* (EN1) vs *P. verrucosum* (A1)*;* D = *Burkholderia cepecia* (EN4) vs *A. niger* (B1); E = *Burkholderia cepecia* (EN4) vs *P. verrucosum* (A1); F = *Novosphingobium* sp. (EN7) vs *A. versicolor* (D15); G = *Novosphingobium* sp. (EN7) vs *P. verrucosum* (A1)]

Figure 2: Antimicrobial activity of endophytes against several mycotoxin-producing fungal strains

An antifungal assay of the endophytes revealed that *Trichoderma harzianum, Dichotomopilus erectus, Talaromyces verruculosus, Burkholderia cepecia* and *Novosphingobium* sp. were the five endophytes with positive activity (Table 2 and Fig. 2). *Trichoderma harzianum* and *Burkholderia cepecia* were the two fungal and bacterial endophytes with the greatest activity against the toxigenic fungi. The other endophytes did not exhibit activity against the pathogens.

Evolutionary relationships of endophytic isolates

The inferred evolutionary relationship between the fungal taxa and bacterial taxa isolated from maize roots in the study was presented in Figs 3 and 4. The phylogenetic tree is an unrooted tree without a common ancestor for the bacterial strain. *Burkholderia cepecia* and *Novosphingobium* sp. are divergent and not closely related to the other bacterial strains in the study. Fig. 4 shows that the common ancestor is the taxon *Talaromyces*. The bootstrap value showed a high probability of genetic change for all the fungal strains isolated. The common ancestor of the sister taxa *Clonostachys rosea, Trichoderma* sp. and *Trichoderma harzianum* lies with the diversity of the *Dichotomopilus erectus strain*.

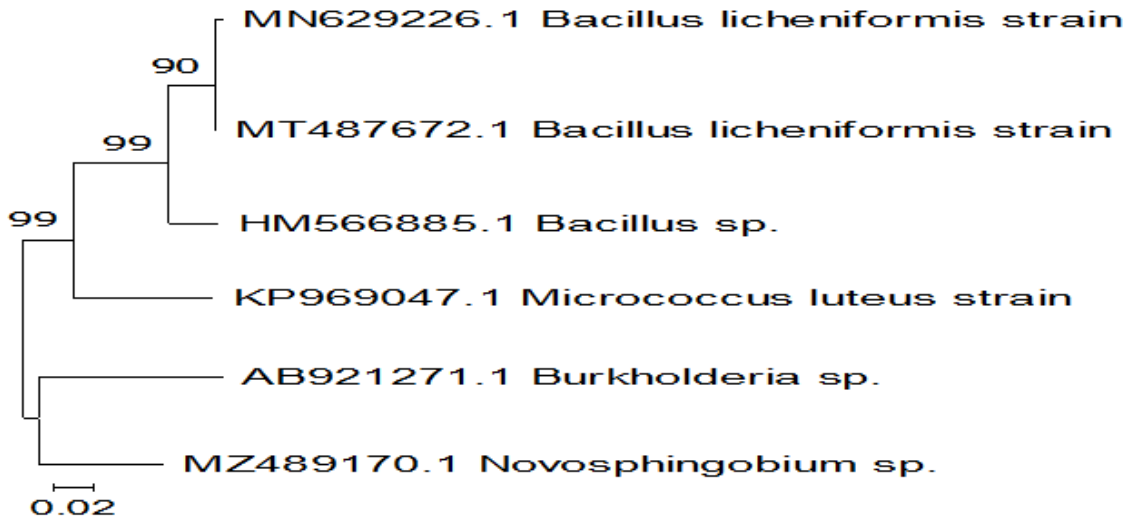


Figure 3: Phylogenetic analysis of the 16SrRNA gene by the maximum composite likelihood method

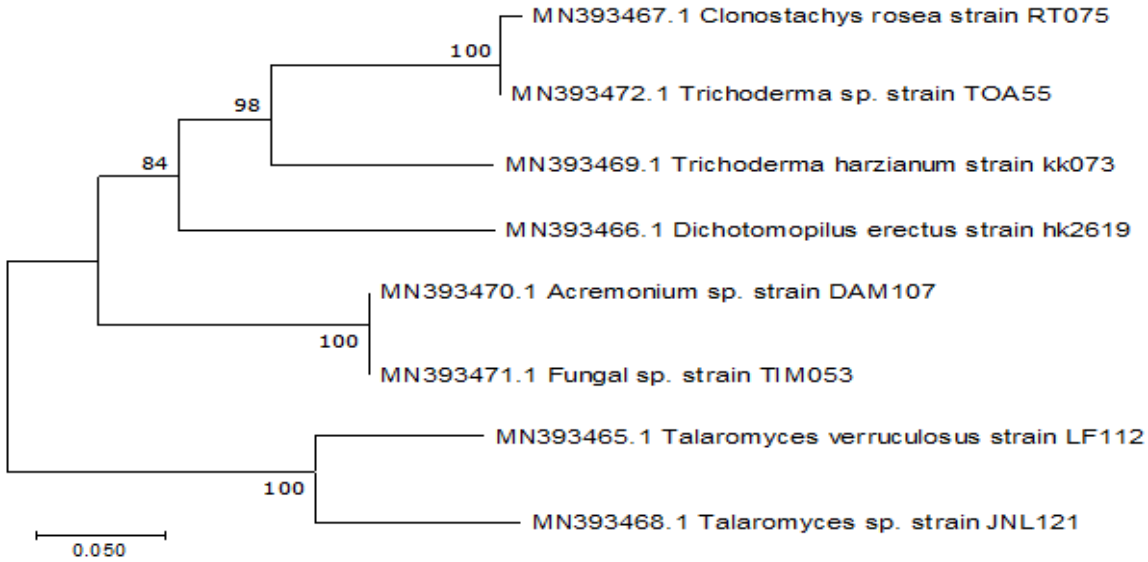


Figure 4: Phylogenetic analysis of the 16SrRNA ITS gene by the maximum composite likelihood method

Endophytic microorganisms benefit host plants by helping them overcome stress conditions caused by the environment, pathogens, natural forces and competitors for resources. In maize plants, endophytes promote development, protect against infection and increase root mass [8, 9]. The low number of fungal and bacterial endophytes isolated could be attributed to the plant roots sampled, climatic and ecological factors, geographical location, humidity and application of fertilizer and pesticides. The production of metabolites by endophytes is dependent on the state of the host plant, species of plant and endophyte, geographical location, season and climatic conditions [10]. Endophytes produce metabolites belonging to the carboxylic acid, nitro compound, aldehyde, ester, ketone, amide, phenol, and alcohol classes, which confer host resistance to pathogen attack, regulation of gene expression of metabolites and growth, and inhibition of abiotic stresses [2, 11].

The endophytes isolated in this study have been reported to confer different advantages to plants. The antifungal activities exhibited by the endophytes were more pronounced in the fungal strains. The antifungal activity of *Trichoderma harzianum* was greatest, which agreed with the finding reported by Pierre *et al.* [12]. The endophyte competes against pathogens, causes mycoparasitism, produces metabolites and enzymes or induces the defense system in the plant host to inhibit pathogen infection [13]. *Trichoderma* species produce trichodermin, trichothecinol A and koningiopisin C, which inhibit fungal infection in plants [14, 15]. *Bacillus licheniformis* and *Clonostachys rosea* have been reported as biocontrol agents [16, 17]. The finding that *Clonostachys rosea* is a biocontrol agent with antifungal potential was negated by the results of this study. The poor antifungal activity or lack of antifungal activity of endophytes might be due to the nature of the metabolites produced and the composition or quantity of the metabolites produced.

The cost associated with the production of some metabolites, reproducibility under a controlled environment and difficulty in their extraction necessitate the exploration of new metabolites. Xingyuan *et al*. [18] reported that metabolites produced by endophytes result from biosynthesized materials from plants. *B. cepacia* showed antifungal effects, which can be attributed to the activity of antioxidants (glutathione and ascorbic acid) produced by the bacteria. Bacteria are also known to relieve stress in host plants [19]. *T. verruculosus* displayed inhibitory activity against fungal pathogens, which is associated with the production of α-glucosidase, funicone, 9,14-epoxy-11-deoxyfunicone and talaron, which give varying levels of antifungal inhibition [1, 20]. The genus also produces quinones, which are antioxidants with antibacterial characteristics. *Clonostachys rosea* produces antibiotic compounds responsible for the antifungal activity recorded in the study [21].

Burkholderia cepecia and *Novosphingobium* sp. are likely to belong to a different group of bacteria because their positions are divergent, with significant genomic differences from those of the other taxa. Among the bacterial species isolated, *Bacillus licheniformis* strains are the most recent. They have a common ancestor likely found in the diversity of the taxon *Micrococcus luteus*. The *Bacillus* taxa evolved, while *Micrococcus luteus* diverged due to evolutionary lineage changes over time. The *Talaromyces* strains were the most evolutionarily distant fungal isolates. The two strains are divergent with significant genomic differences. *Clonostachys rosea* and *Trichoderma* sp. are the most recent strains among the fungal taxa with significant genomic characteristics and similarities. A more distant relationship occurred among the *Talaromyces* taxa than among the other fungal taxa identified in the present study. This means a greater level of evolutionary modification or accumulation of hereditary (genetic) and structural changes among the taxa.

The diversity of the endophytic community greatly determines the composition of metabolites produced by the host plant. The type and quantity of metabolites produced are factors related to the type of endophyte that resides in the plant. The diversity of endophytic strains was greater in the roots than in the roots of other plant parts, which supports the choice of sample collection from the root system of maize plants [22]. The secondary metabolites produced are low-molecular-weight compounds in various amounts and quantities depending on the strain and stress factor (anthropogenic or natural). The challenge of mass production of metabolites from endophytes persists due to the difficulty of growing *in vitro* [23]; however, some of these methods are organ specific, while others are plant specific and involve complex mutualistic interactions with the host.

Conclusion

This study identified fungal and bacterial strains with antifungal properties attributable to the metabolites produced by the endophytes. The antifungal activity of the endophytes was not closely related to that of the *Talaromyces* species obtained in the present study. Repeated clustering of genes over time might be responsible for the high diversity observed in metabolite production and subsequent inhibition of toxigenic fungi by endophytes.

Conflict of interest: There is no conflict of interest among the authors.

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