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Molecular Characterization of Potential Crop Pathogens Associated with Weeds as Endophytes in Uniilorin Plantations, Nigeria

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Abstract:

Crop diseases are usually caused by inoculum of pathogens which might exist on alternate hosts or weeds as endophytes. These endophytes, cum pathogens, usually confer some beneficial attributes to these weeds or alternate hosts from protection against herbivores, disease resistance, stress tolerance to secondary metabolites production. This study was therefore carried out to isolate potential crop pathogens which exist as endophytes on weed species in the University of Ilorin plantations. Green asymptomatic leaves were collected from 10 weed species across the plantations, and processed for their endophytic fungi isolation. Isolates were purified into pure cultures and used for molecular identification using the internal transcribed spacer (ITS) region of the ribosomal DNA. Phylogenetic analysis of the fungal sequences using MEGA software revealed 9 fungal genera belonging to 13 species, with species in the genera *Curvularia*, *Epicoccum* and *Daldinia* occurring in more than one weed species, while other genera such as *Alternaria*, *Fusarium*, *Chaetomium*, *Macrophomina*, *Arthrinium* and *Phomopsis* occurred in just one weed species each. *Daldinia eschscholtzii* was isolated in this study as an endophyte from *Loudetia arundinacea* for the first time. This plant is very abundant in Nigeria and Africa where it is used majorly for thatching and feeding livestocks. This also represents the first endophytic fungi from the genus *Loudetia*. Potential relationship between the occurrences of these fungi as endophytes and as pathogens are discussed. These discoveries represent the first large-scale molecular identification and several first reports of endophytes from these weed species. These results also represent the first records of some of these fungi in Nigeria.

Keywords: *Curvularia*, ITS (Internal Transcribed Spacer), *Loudetia arundinacea*, New records, Phylogenetic analysis.

Introduction:

Just recently,[1]has raised provocative questions about the importance and potential role of schizotrophic endophytes in natural ecosystems. Schizotropism (split nutritional strategy) is the "opposite lifestyles" of fungal species (for example *Daldinia mutila*, *Sclerotium sclerotinum* and *Colletotrichum* spp.) on the endosymbiont–pathogen continuum in different host plants and there will likely be more microbes with these characteristics in nature. An endophyte's life cycle includes both vertical transmission through host seeds and horizontal transmission through infective spread using fungal spores [2]. These endophytes may alter competitive abilities of host individuals and improve host fitness under stress, but may also become pathogenic in other plants [2].

Weeds are unwanted plants that grow along with cultivated plants [3]. The weeds grow aggressively in the field and most of them are characterized by possession of seeds with different dormancy periods, high adaptability to the growing environment, self-compatibility in terms of pollination and easy dispersal [4]. These weeds are very detrimental to agronomical operations as they compete with crops for nutrients, space, light and water, reduce crop yield, interfere with workability

of farm machinery, harbour pathogens (including endophytes cum pathogens) and release allelopathic substances. The overall effect of these unwanted plants is one of the factors that are not only responsible for economic losses in agricultural production [5] but also causes damage to the environment [6].

Positive plant-microbe interaction is clearly manifested in the relationship between weeds and endophytic fungi. Endophytes make up part of the endobiome of plants including weeds [7,8] where they colonize their internal and living tissues without harming the host. They live mutualistically with the host. As the plant provide essential nutrients for the fungi, the fungi produce metabolites that confer chemically induced defense against external attack by animals, insects or other microbes, enhance its tolerance to environmental stress such as drought tolerance and heavy metal tolerance and positively influence plant growth and development [9]. Much attention has been shown to the control of weeds in the field and extensive work has been done in this regard. However, there is insufficient information available on the significance of weeds with their associated endophytic fungi which may even account for their therapeutic roles in traditional medicine and pharmaceutical industries.

Despite the fact that weeds are harmful and detrimental, they perform beneficial roles in the lives of man and livestock. Weeds provide vegetative cover to prevent both wind and water erosion, serve as food for man and livestock, help in recycling of nutrients in the soil, provide shelter for beneficial insects that partake in pollination, use as ornamental plants and biopesticides, and contain useful secondary metabolites that make them indispensable in tradition medicine and pharmaceutical industries. Weeds in herbal medicine are taken as relaxants, painkillers, tonic antiseptics, for digestive purposes and for skin disorders. The leaf of *Ageratum conyzoides* is efficacious in wound healing and the juice from the leaf is used to cure skin diseases [10]. *Chromolaena odorata* leaf is used singly as cough remedy and in combination with guava leaf for the treatment of malaria [11]. However, medicinal properties of plants are majorly attributed to their phytochemicals and synergy of secondary metabolites being produced by endophytes [12].

This work, therefore, aims to unveil the dominant endophytic fungi in selected weeds which may have multi-faceted significance as endophytes cum pathogens, or as resources for beneficial secondary metabolites production in cultivated plantations in the University of Ilorin.

Materials and Methods:

Sample collection

The sample collection sites were four plantations in the University of Ilorin campus, latitude 80, 29'N and longitude 40, 35'E, 320 m above sea level based on GPS readings Fig. 1. The vegetation is composed of Guinea Savanna Ecological Zone with rainfall which falls between May and September.

Green asymptomatic leaf samples were collected from plants regarded as intruding weeds in the plantations Tab. 1. Samples were collected into sterile polythene bags, labeled appropriately and transported to the laboratory for endophytic fungi isolation.

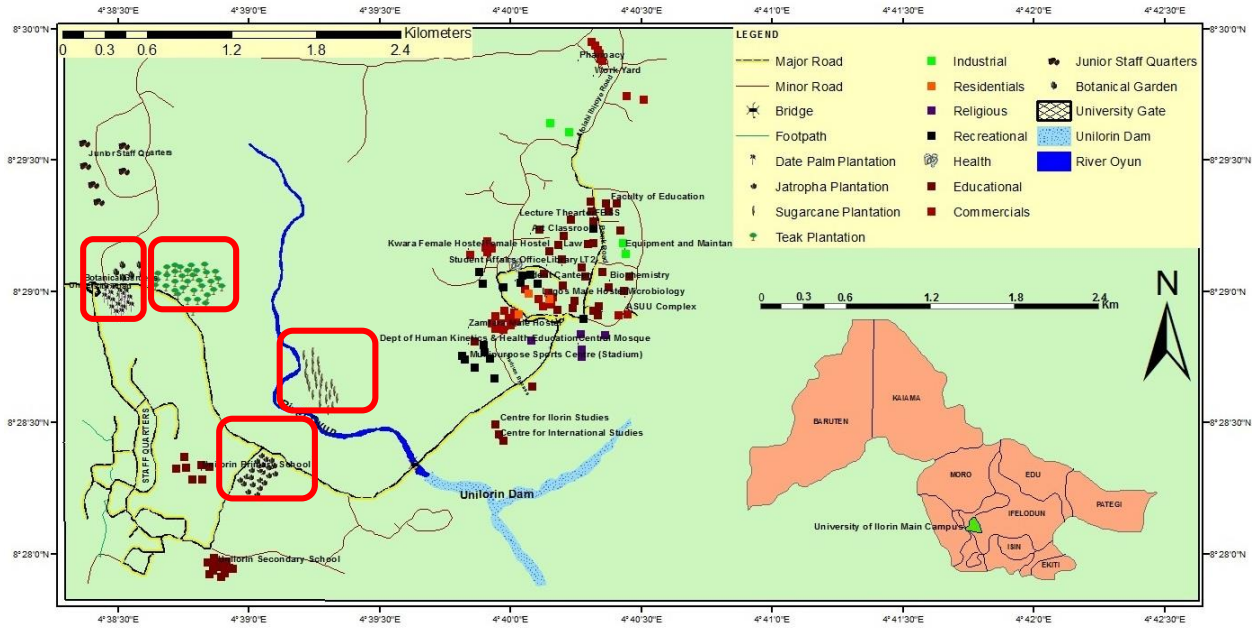

Figure 1. Map of University of Ilorin showing the plantations (in red rectangles) where samples were collected (Source: Unilorin, 2020).

Isolation of endophytic fungi:

Endophytic fungi isolation was done following the method of [13]. Briefly, the leaves were cut into small segments of 2 cm^2, rinsed in sterile distilled water followed by 30 sec in ethanol, 1 min in 10 % hypochlorite (bleach), 30 sec in ethanol and then five rinses in sterile distilled water. The leaves were allowed to dry out before plating on potato dextrose agar and subsequently obtaining pure cultures after 1-2 weeks.

Genomic DNA Extraction, PCR (Polymerase Chain Reaction) amplification and Sequencing.

Mycelia from pure cultures were scrapped with sterile scapel into ZymoResearch DNA extraction kit (California, USA) tube and the extraction was done following the manufacturer's instruction. PCR amplification and sequencing were done at the Africa Genomic Inqabba Biotec in South Africa using the primer pair ITS 5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS 4 (5′-TCCTCCGCTTATTGATATGC-3′) [14]. The PCR mixture was prepared by adding 12.5 µl of Taq 2X PCR master mix, 1 µl of each primer (10 µM) (ITS 5 and ITS 4), 9.5 µl of double-sterilised distilled water (ddH2O) and 1 µl of the DNA template. For the control reaction, ddH2O was used as the template instead of the DNA. The PCR programme used was 2 mins at 94 ˚C, followed by 35 cycles at 94 ˚C for 1 min, 55 ˚C for 1 min, 72 ˚C for 1 min and final extension at 72˚C for 10 mins. Sanger sequencing of the PCR products were carried out in an ABI PRISM sequencer at the Africa Genomic Inqabba Biotec in South Africa using the same primers. The DNA concentration, purity and PCR size were documented.

Phylogenetic Analysis

Forward and reverse sequences obtained from the sequencing were merged to obtain the consensus sequence using SeqTrace version 0.9.0 [15] and the consensus used for similarity search using BLAST-n on the GenBank website (http://www.blast.ncbi.nlm.nih.gov). Highly similar sequences from reputable laboratories were downloaded from GenBank for phylogenetic analysis. Aliview version 1.17-beta1 software [16] was used for the alignment and the phylogentic tree was constructed in MEGA using maximum likelihood.

The tree was rooted with *Diaporthe vaccinii* (MF380877). Sequences of the newly identified fungi were deposited in GenBank to obtain their accession numbers Tab. 1.. Stock cultures of the identified endophytes were stored in the Microbial collections of the Department of Plant Biology, University of Ilorin, Nigeria.

Results:

Molecular identification and phylogenetics

In this study, dominant fungal endophytes were isolated using PDA from 10 weed plants. The isolated fungi were identified morphologically based on their culture characteristics which resulted in 13 fungal species from 9 genera. The DNA purity and concentration ranged from 1.7 – 1.8 and the PCR products ranged between 600 – 700 bps. Phylogenetic analysis of the isolated fungal DNA sequences using maximum likelihood in MEGA 7 showed the various relationships of the isolated fungal endophytes with their close relatives supported by high bootstrap values Fig. 2.

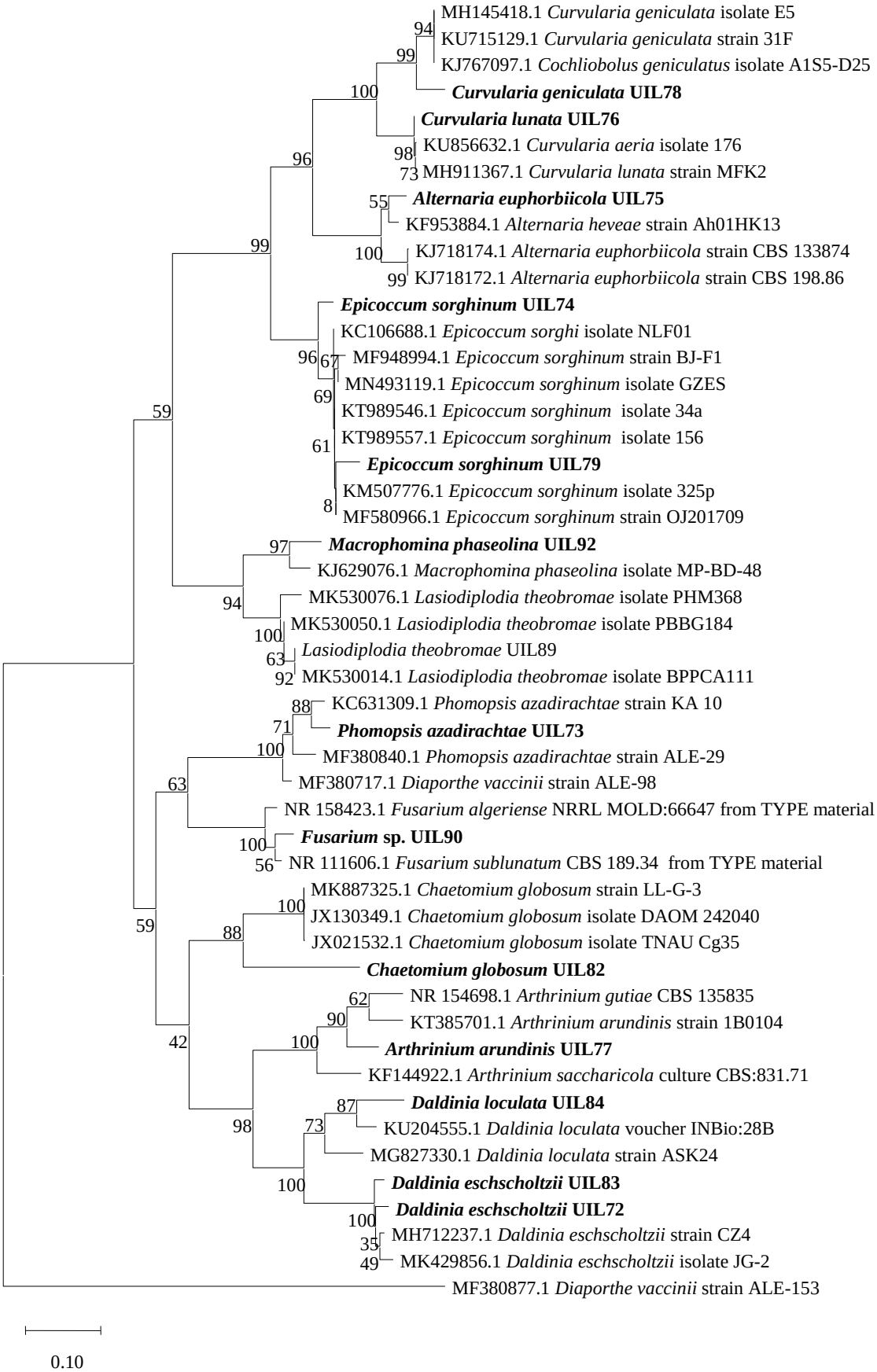

Figure 2. Phylogenetic tree shows (in bold) the molecular identification of the isolated endophytic fungi from this study. Maximum likelihood bootstrap values are shown on the tree branches.

Two isolates were identified as *Epicoccum sorghinum*, another two as *Daldinia eschscholtzii*, one each as *Alternaria euphorbiicola*, *Curvularia lunata*, *Curvularia geniculata*, *Macrophomina phaseolina*, *Arthrinium arundinis*, *Daldinia loculata*, *Chaetomium globosum* and *Phomopsis azadirachtae* Fig. 3. However, only *Fusarium* sp. was unable to be identified to species level.



Figure 3. Morphology of the isolated fungal isolates A. Front view of *Curvularia geniculata* B. Reverse view of *Curvularia geniculata* C. Front view of *Epicoccum sorghinum* D. Reverse view of *Epicoccum sorghinum* E. *Daldinia eschscholtzii* F. *Fusarium* sp.

Table 1. Endophytic fungi isolated from different weed species and their GenBank accession numbers.

S/N	Weed species	Plantation	Fungi Isolated	GenBank Accession Number
1	*Loudetia arundinacea*	Jatropha	*Daldinia eschscholtzii*	MT875384
2	*Chromolaena odorata*	Sugarcane	*Epicoccum sorghinum*	MT875375
3	*Chromolaena odorata*	Sugarcane	*Alternaria euphorbiicola*	MT875377
4	*Chromolaena odorata*	Sugarcane	*Epicoccum sorghinum*	MT875376
5	*Bridellia ferruginea*	Sugarcane	*Chaetomium globosum*	MT875386
6	*Ageratum conyzoides*	Sugarcane	*Arthrinium arundinis*	MT875382
7	*Ageratum conyzoides*	Sugarcane	*Curvularia geniculata*	MT875379
8	*Boerhaavia diffusa*	Sugarcane	*Curvularia lunata*	MT875378
9	*Desmodium trifolium*	Sugarcane	*Daldinia eschscholtzii*	MT875385
10	*Etanda africana*	Date palm	*Daldinia loculata*	MT875383
11	*Gomphrena celosioides*	Jatropha	*Fusarium* sp.	MT875388
12	*Centrosema pubescens*	Date palm	*Macrophomina phaseolina*	MT875381
13	*Aspilia africana*	Date palm	*Phomopsis azadirachtae*	MT875387

Diversity and occurrence (distribution) of the fungal isolates

Most of the weed plants used for the isolation occurred as perennials in plantations in the University of Ilorin.

Two [2] endophytes; *Daldinia eschscholtzii* and *Fusarium* sp. were isolated from weeds in Jatropha plantation, three [3]; *D. loculata*, *Macrophomina phaseolina* and *Phomopsis azadirachtae* isolated from weeds in Date palm plantation and seven [7]; *D. eschscholtzii*, *Epicoccum sorghinum*, *Alternaria euphorbiicola*, *Chaetomium globosum*, *Arthrinium arundinis*, *Curvularia lunata* and *C. geniculata* from weeds in Sugarcane plantation.

Two fungal genera *Curvularia and Daldinia* were each isolated as endophytes in two and three different weed species respectively while other genera occurred in just one plant species each Tab. 1. *C. lunata* and *C. geniculata* were isolated from *Boerhavia diffusa* and *Ageratum conyzoides* respectively; *Daldinia eschscholtzii* isolated from both *Loudetia arundinacea* and *Desmodium trifolium*; while *D. loculata* was isolated from *Etanda africana*.

Two plant species *Chromolaena odorata* and *Ageratum conyzoides* had two different fungal endophytes isolated from them each, while other plants had only one each. *Epicoccum sorghinum* and *Alternaria euphorbiicola* were isolated from *C. odorata*, while *C. geniculata* and *Arthrinium arundinis* were isolated from *A. conyzoides* respectively.

Discussion:

The use of DNA sequences for identifying fungal endophytes has been a very useful and precise molecular tool in fungal biology with the ITS region being used as the primary barcode for identification of fungi [17]. This study therefore used the rDNA genes for the identification of isolates from the weeds species. It will be observed that all fungal isolates in this study identified the species level except for *Fusarium* sp. This is due to the close relationships between *Fusarium* species in the ITS regions of their DNA thus resulting in low resolution of species at this level. [18] reported the use of TEF1 and β-tubulin regions for better identification of *Fusarium* to the species level. [19] noted that the GenBank database is sufficiently robust for endophytic fungi molecular identification. From the phylogenetic tree, it can be observed that the isolated endophytes were classified into two main clades, majorly with high bootstrap support. Each sub-clades was also well supported with high bootstrap value which indicates a well resolved phylogenetic tree, with different name in same sub-clade indicate synonyms

The isolation of endophytes from these weed species from the University of Ilorin plantations represent an effort to characterize the fungal species associated with these plants, thereby establishing a host-endophytes relationship blue-prints for these plants. The selected weed species were occurring dominantly in the plantations meaning that they can serve as a stable source for the isolation of the identified fungal endophytes and as well as a potential source of pathogens in the plantations by transiting the endosymbiont–pathogen continuum.

In Jatropha plantation where *D. eschscholtzii* and *Fusarium* sp. were isolated, [20] have reported *Fusarium* sp. causing die-back disease on Jatropha plants. However, *Daldinia* spp. has not been recorded in Jatropha plantations.

In Date palm plantation where *Phomopsis* was isolated from the weed *Aspilia africana*, Daldinia from *E. africana* and *Macrophomina* from *C. pubescens*, fruit rot and leaf spot has been reportedly caused by *Phomopsis* on date palm crops [21].On the otherhand, *Daldinia* and *Macrophomina* has not been recorded as capable of causing diseases on Date palm.

From the endophytes isolated in Sugarcane plantation, two species of *Curvularia* was isolated from two different weeds. Other endophytes include *D. eschscholtzii*, *E. sorghinum*, *A. euphorbiicola*, *C. globosum* and *Arthrinium arundinis.* [22] have reported *Curvularia* spp. to be one of the prevalent pathogens of Sugarcane. Similarly, *Chaetomium* sp. has been reported as a pathogen of sugarcane in Cameroon [23], *Alternaria* sp. in China [24] and *Arthrinium* sp. in China [25] respectively. On the other hand, *Daldinia* spp and *Epicoccum* spp. have not been reported as pathogens on Sugarcane, however, *Epicoccum* sp. has been reported to enhance the growth of Sugarcane in Brazil [26].

Among the plant species used for isolation, *Chromolaena odorata* and *Ageratum conyzoides* both had more than one fungal endophytes associated with them. This signifies their capacity to host different fungal species which can be linked with their high dominance and rapid growth in the field, as endophytes have been known to increase plant growth and protection against biotic and abiotic stresses [27]. Also, [28–30] have also reported a similar trend of multiple endophytes associated with the leaves, stem and roots of *Myracrodruon urundeuva*, *Distylium chinense* and *Paullinia cupana* respectively.

The two fungal genera *Curvularia* and *Daldinia* that were isolated as endophytes in more than one weed species in this study have also been reported from several plant hosts. *Curvularia* species are able to colonize many host plants due to their ability to produce diverse secondary metabolites for greater niche exploitation [31]. *Curvularia geniculata* isolated from *C. odorata* has been reported to promote plant growth by the production of indole-acetic acid (IAA) [32] and this can be linked to the aggressive pattern of growth of *C. odorata* in the field. As well, *Daldinia eschscholtzii* have been reported to produce several metabolites with antimicrobial properties [33] thus, contributing to the better growth and medicinal uses of their host plants. These two fungi have also been reported occurring frequently and concurrently on sporulating rice in Thailand [34] thus further signifying their potentials as biologically active fungal species. *Alternaria* isolated from *C. odorata* also has biological activities against human tumor cells and amastigotes of *Leishmania amazonensis* parasite [35].

Daldiniia eschscholtzii isolated in this study as endophytes from *Loudetia arundinacea* and *Desmodium trifolium* for the first time. *L. arundinacea*e is very abundant in Nigeria and Africa where it is used majorly for thatching and feeding livestocks while *D. trifolium* is used for treating malaria, dysentery and for other medicinal purposes [36]. This also represents the first endophytic fungi from the genus *Loudetia*.

[37,38] isolated endophytic fungi from *C. odorata* but *Epicoccum sorghinum* and *Alternaria euphorbiicola* were not reported. However, *E. sorghinum* and *A. euphorbiicola* were severally isolated in this study from *C. odorata* which can be influenced by site characteristics and possibly inherited from surrounding plants [28,39]. In addition, *E. sorghinum* has been reported as a pathogen in seeds of *Sorghum bicolor* [40], as pathogen on Taro leaves [41], and as endophyte in *Caesalpinia echinata* [42], so as *A. euphorbiicola* was isolated as pathogen in *Euphorbia heterophylla* [43], *E. prunicola*, *E. heterophylla* and *E. marginata* [44].

These endophytes may become pathogenic or remain endophytic on their initial host or nearby plants depending on the circumstances such as the host physiological condition, the environmental conditions and nearby plants' condition. These factors could influence where an endophyte is placed on the endosymbiont-pathogen continuum [2]. Suboptimal environmental conditions may pre-dispose a host plant resulting in disease and subsequent transfer of inoculums to nearby plants depending on the prevailing environmental condition [45]. Although, transition of endophytes on the endosymbiont-pathogen continuum have not been studied thoroughly, this study shows that these endophytes' transition can occur in the field.

No endophytic fungi have been earlier reported from *Bridelia ferruginea*. The isolation of *C. globosum* constitute the first endophyte from this plant. Similarly, *Chaetomium globosum* which was isolated from *Bridelia ferrunginea* have been previously isolated as endophyte from *Gossypium hirsutum* and *Salvia miltiorrhiza* [46]. *Curvularia lunata* which was isolated from *Boerhavia diffusa* have however been isolated from *Cymbopogon caesius* [47], *Lippia* sp. [48], wild banana [49] and *Paepalanthus chiquitensis* [50] but not yet from *Boerhavia diffusa*, thus this represents the first record of this endophyte from *B. diffusa*. [51] isolated 15 fungal endophytes from *B. diffusa* in India which includes *Chaetomium* sp., *Colletotrichum* sp., *Pestalotiopsis* sp., *Phomopsis* sp., *Phyllosticta* sp., *Alternaria* sp., *Aspergillus* sp., *Curvularia* sp., *Fusarium* sp., and *Nigrospora* sp. but not *Curvularia lunata*. The recent identification of *C. lunata* in this study can therefore be attributed to the use of molecular techniques which is an improved method for fungal identification. *B. diffusa* leaves have been used in local medicines [52,53] but detailed study on its endophytic fungi has been lacking.

Similar studies to ours have been carried out by [54] on the molecular identification of

endophytic fungi from leaves of *Sapindus saponaria* using the ITS region which resulted in 13 isolates including the genera *Cochliobolus*, *Alternaria*, *Curvularia*, and *Phomopsis*. Furthermore, [19] isolated the genera *Phomopsis*, *Diaporthe*, *Dothideomycete*, and *Cordyceps* from leaves of the medicinal plant *Trichilia elegans*.

One of the most interesting features of these endophytic fungi is their immense diversity. Globally, endophytic fungal diversity is estimated at more than one million species, many of which inhabit different parts of individual plants [55] including the leaves, roots and stems.

The tropical regions of the world have the highest diversity of endophytic fungi as a result of the high plant diversity in those regions, therefore, exploration and exploitation of the endophytic fungi associated with different plant species as done in this study are important for the assessment of global fungal ecology and for their biotechnological potentials.

Conclusion:

In conclusion, it is evident that the occurrences of 13 endophytic fungal species on 10 different host plants in the University of Ilorin commercial plantations might serve as a potential risk for fungal diseases of the crops grown in the plantations. This study further reveals several first records of endophytic fungi inhabiting some of the studied host plants. These discoveries will serve as a reference point for further exploration of molecular identification of fungi, actual pathogenicity of those fungi and exploitation of their biotechnological applications.

The occurrence of these fungal species as endophytes in these weed species is also being considered as an exploratory opportunity for drug discovery through their secondary metabolites.

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Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee in University of Ilorin.

Authors' contributions statement:

LAA, GT, AKA, OBU, OGS, ASB and AAA concepted and designed the research. All authors collected the samples, did the sample analyses and approved the final copy of the manuscript. LAA, GT and OGS wrote and edited the manuscript. LAA and GT did the sequence analyses. LAA did the phylogenetic analysis.

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التوصيف الجزيئي لمسببات امراض المحاصيل المحتملة المرتبط بالأدغال كعائل نباتي داخلي في مزارع جامعة إيلورين، نيجيريا

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الخلاصة:

عادة ما تحدث أمراض المحاصيل عن طريق لقاح من مسببات الأمراض التي قد توجد على عوائل بديلة أو حشائش مثل عائل نباتي داخلي. عادة ما تمنح هذه العوائل النباتية الداخلية ، مسببات الأمراض ، بعض السمات المفيدة لهذه الأعشاب أو المضيفات البديلة من الحماية ضد العواشب ، ومقاومة الأمراض ، وتحمل الإجهاد لإنتاج مركبات الايض الثانوية. لذلك أجريت هذه الدراسة لعزل مسببات الأمراض المحتملة التي توجد كعوائل نباتية داخلية في أنواع الادغال في مزارع جامعة إيلورين. جمعت الأوراق الخضراء عديمة الأعراض من 10 أنواع من الادغال عبر المزارع ، وتم معالجتها لعزل الفطريات من العائل النباتي الداخلي. تم تنقية العزلات إلى مستنبتات نقية واستخدمت في التعريف الجزيئي باستخدام منطقة المباعد المنسوخة الداخلية **(ITS)**للحمض النووي الريبوسومي. كشف التحليل الوراثي للتسلسل الفطري باستخدام برنامج **MEGA**عن 9 أجناس فطرية تنتمي إلى 13 نوعًا ، مع وجود أنواع في الأجناس **Curvularia**و **Epicoccum**و **Daldinia**في أكثر من نوع واحد من الادغال ، بينما توجد أجناس أخرى مثل **Alternaria**و **Fusarium**و **Chaetomium**و **Macrophomina**و **Arthrinium**و **Phomopsis**في نوع واحد فقط من الادغال لكل منهما. تم عزل **Daldinia eschscholtzii** في هذه الدراسة باعتباره عائل نباتي داخلي من **Loudetia arundinacea**لأول مرة. هذا النبات متوفر بكثرة في نيجيريا وإفريقيا حيث يتم استخدامه بشكل رئيسي بتغطية السقوف بالقش وتغذية المواشي. يمثل هذا أيضًا أول فطريات في عائل نباتي داخلي من جنس **Loudetia..** تمت مناقشة العلاقة المحتملة بين حدوث هذه الفطريات باعتبارها عائل نباتي داخلي ومسببات الأمراض. تمثل هذه الاكتشافات أول تحديد جزيئي واسع النطاق والعديد من التقارير الأولية عن عوائل نباتات داخلية من هذه الأنواع من الادغال. تمثل هذه النتائج أيضًا التسجيل الأولي لبعض هذه الفطريات في نيجيريا.

الكلمات المفتاحية: Curvularia، ITS فاصل النسخ الداخلي، Loudetia arundinacea ، تسجيل جديد ، الشجرة التطورية.