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Screening extracts of fungal endophytes isolated from *Allophylus abyssinicus* (Hochst. Radlk.) for control of bean anthracnose

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Common bean is consumed in large quantities globally but it is highly susceptible to *Colletotrichum lindemuthianum*, leading to 90 to 100% yield losses. This study investigated the bioactivity of secondary metabolites from *Allophylus abyssinicus* fungal endophytes against *C. lindemuthianum*, causing bean anthracnose. A total of 37 fungal endophytes were isolated from the leaves, bark and roots of *A. abyssinicus* and 20% identified by ITS-rDNA sequence analysis as *Aspergillus hancockii, Penicillium christenseniae, Penicillium atrosanguineum* and *Penicillium manginii*. Thirty-three of the endophytes were active against *C. lindemuthianum* in the dual culture assay with the highest inhibition being 82.6% (*A. hancockii*). Two of the most active endophytes (*A. hancockii* and *P. christenseniae*) were fermented on rice media and their methanol extracts partitioned between ethyl acetate and hexane. *Aspergillus hancockii* crude hexane extract had the highest inhibition (19.0±1.7mm at 100 mg/ml) against the pathogen under the bioassay screening and 6.3±3.8 mm at 6.25% against *C. lindemuthianum* under the minimum inhibitory concentration (0.625 mg/ml) screening. Chemical screening of the extracts revealed presence of alkaloids in *A. hancockii* and *P. christenseniae*; sterols, triterpenes and coumarins in *A. hancockii*. These results indicated that fungal endophytes from *A. abyssinicus* are a source of active compounds that can be used to control *C. lindemuthianum* affecting common bean.

Key words: *Allophylus abyssinicus*, Antifungal activity, *Colletotrichum lindemuthianum*, fungal endophytes, secondary metabolites.

INTRODUCTION

The common bean (*Phaseolus vulgaris* L.) ranks as the most consumed legume globally contributing to food security and solving malnutrition (Siddiq et al., 2022). It is a source of protein, resistant starch, dietary fibre,

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antioxidants, minerals and vitamins (Aguilera et al., 2011; Pedrosa et al., 2015). Common bean is highly affected by anthracnose caused by *Colletotrichum lindemuthianum* which is seed-borne (Gillard and Ranatunga, 2013) leading up to 100% yield loss in susceptible cultivars (Mohammed, 2013). Disease symptoms occur as dark brown sunken, circular to elliptical lesions that may girdle the stem and on pods. They start as small reddish-brown, slightly sunken circular spots that turn to larger, dark sunken lesions distorting the pods (Jadon et al., 2020). Anthracnose conducive developmental conditions include temperatures of about 17°C, relative humidity above 92% and soil pH of 5.8-6.5 (Padder et al., 2017).

C. lindemuthianum conidium comes into contact with the susceptible bean plant leaf, stem or pod, adheres to the plant cuticle, and germinates (Alkemade et al., 2021). In humid conditions, the conidium germinates and produces an appressorium used for epidermal cell penetration (Jadon et al., 2020). The appressorial surface adhering to the cuticle is flattened and a pore form where the infection peg emerges and pierces the bean leaf cuticle and cell wall. It then penetrates into the host epidermal cell (Chethana et al., 2021). Various enzymes such as cutinase, oxidase and lipases are secreted from the infection peg that degrade plant cuticle and wax layers (Cook and Decuzzi, 2021). The infection peg protrudes from the appressorium, penetrate the cell wall where infection hyphae grow and develop into infection vesicles. *Colletortichum lindemuthianum* is a hemibiotrophic fungus (Dubrulle et al., 2020), spending part of the infection cycle as a biotroph and the other as a necrotrophy. The pathogen establishes an initial asymptomatic biotrophic phase during the infection process, invading its host's tissues (*P. vulgaris*) undetected (Newman and Derbyshire, 2020). Bean anthracnose is a highly destructive disease affecting dry bean production and significantly reduces both seed yield and quality of dry beans under favorable conditions resulting in reduced marketability of the crop.

Various methods have been used to control bean anthracnose such as use of resistant cultivars and chemical control, with the pathogen overcoming the resistance in some commercial cultivars (Rodriguez-Guerra et al., 2003). Biocontrol using beneficial microbes is a much better option compared to chemical control (Medeiros et al., 2012). These beneficial microbes include endophytes which live symbiotically within plant tissues and cause no disease symptoms (Abbamondi et al., 2016). These endophytes inhibit plant diseases by inducing resistance in the host plant, competition for food and space, and also antibiosis (Medeiros et al., 2012).

Allophylus is the largest genus in the Sapindaceae family with about 255 plant species distributed worldwide. Roots and twigs of *Allophylus africanus* are used to treat gastritis, hookworm, venereal diseases, burns, sores and fever in west Africa (Lewis et al., 2003). Roots of *Allophylus ferrugineus* var. *ferrugineus* (Sapindaceae) are used to treat gastritis, coughing, round worms and fever in the Shengena forest reserve of Tanzania (Boer et al., 2005). *Allophylus edulis* is a source of sesquiterpenes (Foster et al., 2004), flavonoids, phenolic compounds (Arisawa et al., 1989), tannins, and essential oils (Rasico, 2007). Roots of *Allophylus abyssinicus* treat coughs and rheumatism in Kenya, while leaves are used to treat helminths and the fruits to cure venereal diseases in Ethiopia (Chavan and Gaikwad, 2013). Powdered leaves of *A. abyssinicus* are applied to treat wounds or ingested to treat inflammatory conditions. This study was undertaken to evaluate the efficacy of secondary metabolites of fungal endophytes from *A. abyssinicus* on *C. lindemuthianum* for potential application in biocontrol of bean anthracnose.

MATERIALS AND METHODS

Plant

Fresh stem bark, root and leaves of *A. abyssinicus* were collected from Mt. Elgon Forest which is tropically humid with a latitude of 1°08˙N, longitude 34° 45˙E and an altitude of 4321 m above sea level. The plant was identified by a plant taxonomist (Professor Samuel T. Kariuki) and voucher specimen was deposited at the Department of Biological Sciences, Egerton University.

Fungal endophyte isolation protocol

Endophytic fungi were isolated from fresh bark, roots and leaves of *A. abyssinicus* using the procedure described by Zinniel et al. (2002) with slight modifications. The samples were cleaned under running tap water and immersed in 70% ethanol for 3 min, followed by immersion in 2.5% sodium hypochlorite solution for 3 min. These samples were rinsed three times (1 min each) with sterile double distilled water, blot dried with sterile paper towels and cut aseptically into sections of approximately 1 mm by 4 mm using sterile scalpel blades. The tissues were plated on PDA media amended with Streptomycin Sulphate (100 mg/l), incubated at 25±2°C for 1-4 weeks and monitored daily for fungal growth. Fungal mycelia of endophyte isolates were sub-cultured on PDA media without antibiotic to obtain pure cultures which were identified using molecular techniques described by Landum et al. (2016).

Dual culture assay of fungal endophytes against *C. lindemuthianum*

Antifungal activity of the fungal endophytes was screened using dual culture assay method as described by Stadler et al. (2004). Two weeks old pure fungal endophytes grown on PDA at 25±2°C were used. Mycelial agar plugs about 7 mm were cut from actively growing cultures and inoculated opposite *C. lindemuthianum* approximately 2 cm apart on PDA plates. The plates were incubated at 25°C for 7-21 days and monitored for growth inhibition. Inhibition zones between the endophyte and phytopathogen (C-T) were measured after 21 days and the resulting percentage inhibition determined as follows:

$$L = \frac{C-T}{C} \times 100\%$$

where L = inhibition of radial mycelia or colony growth; C=radial

growth measurement of pathogen in control; T = radial growth measurement of pathogen in the presence of antagonist (Hajieghrari et al., 2008).

The experiment was carried out in triplicate and Nystatin discs used against *C. lindemuthianum* as the positive control, whereas for the negative control *C. lindemuthianum* was left to grow in absence of the fungal endophyte.

Molecular characterization of fungal endophytes

Molecular characterization of the fungal endophytes was done according to the method described by Landum et al. (2016). The endophytes were grown on PDA plates, genomic DNA extracted from mycelia of the pure cultures and characterization done by sequencing of the ITS region (rDNA ITS).

DNA extraction and amplification

The DNA extraction was done using the BIO BASIC EZ-10 Genomic DNA kit following manufactures instruction. About 60 mg of the fungal mycelia obtained from a 3 to 4-day old culture was added to a 1.5 ml screw cap reaction tube containing approximately 6-10, 1.4 mm Precellys Ceramic Beads. The sample was covered with 600 µl Plant Cell lysis buffer (PCB) (Sodium propionate, sodium cacodylate and BIS-TRIS propane in a ratio of 2:1:2) and homogenized using a homogenizer (Precellys 24 lysis and homogenization, Peq lab, Bertin Technologies). Approximately 12 µl of β mercaptoethanol was added to the sample to aid in protein degradation. The sample was vortexed (IKA MS3 Digital) to mix the components and incubated for 25 min at 65°C in a metal block (MTB 250). Chloroform (600 µl) was added to solubilize the proteins and polysaccharide from the DNA. The sample was then centrifuged (5430 R) at 10,000 rpm for 2 min and the upper layer transferred to a clean Eppendorf tube while the rest was discarded. Approximately 200 µl of Binding buffer was added and the mixture vortexed, followed by addition of 200 µl ethanol which was later vortexed to mix. The mixture was transferred into EZ-10 column placed in a 2 ml collection tube and centrifuged at 12,000 rpm for 1 min. The mixture was centrifuged at 12, 000 rpm and the flow through discarded. Wash solution (500 µl) diluted with ethanol was added to the mixture to remove the salts. The mixture was centrifuged at 12,000 rpm for 1 min and the flow through discarded and the column centrifuged again at 12,000 rpm for 2 min to remove any remaining wash solution. The column was transferred into an empty 1.5 ml Eppendorf tube and 70 µl of TE Buffer, pre-warmed to 60°C, added directly at the center of the EZ membrane to increase the elution efficiency. The sample was incubated for 2 min at room temperature to increase the elution chances of the DNA from the membrane. The tube was centrifuged at 12,000 rpm for 2 min to elute the DNA which was stored at 4°C for further analysis.

To a PCR tube, it was added 0.5 µl of forward primer ITS1F (CTTGGTCATTTAGAGGAAGTAA) and 0.5 µl of reverse primer ITS4 (TCCTCCGCTTATTGATATGC), 12.5 µL of the jump start ready mix containing 20 mM Tris-HCl pH 8.3, 100 mM KCl, 3 mM $MgCl_2$, 0.002% gelatin, 0.4 mM dNTPs (dATP, dCTP, dGTP, dTTP), stabilizers, 0.1 unit/mL Taq DNA polymerase and JumpStart antibody. This was followed by 9.5 µl of distilled water and 2 µl of the template DNA to make a total volume of 25 µl of the mixture per sample. For a negative control, 2 µl of distilled water was used instead of DNA template in the reaction mix. The amplification was done in a thermocycler (Eppendorf® Mastercycler® nexus Thermal Cycler) under the following conditions; initial denaturation of 5 min at 94°C, followed by 34 cycles of denaturation for 30 s at 94°C, annealing at 52°C for 30 s and elongation for 1 min at 72°C. A final elongation of 10 min at 72°C was added. The PCR products were pre-stained with midori green dye and resolved in a 0.8% agarose gel. The visualization was done in a UV transilluminator (Nippon Genetics Europe GMbH) and photographs taken.

PCR product purification and DNA sequencing

The amplified PCR products were purified using BIO-BASIC EZ-10 spin column purification kit following the manufacturer's instructions. To adjust the DNA to the binding conditions, 110 µl of buffer 1 was added to the 22 µl PCR product and mixed thoroughly. The mixed sample were then put on the EZ-10 spin column and incubated for 2 min at room temperature. Later, the sample was centrifuged at 10,000 rpm for 30 s and the flow-through discarded. The DNA was washed by adding 500 µl wash solution, centrifuged at 10,000 rpm for 30 s and the flow-through discarded. The same amount of wash solution was added to the sample and then centrifuged at 10,000 rpm for 1 min and the flow-through discarded. The amplified DNA was eluted in a clean 1.5 ml reaction tube by adding 20 µl of elution buffer pre-warmed to 65°C. The sample was incubated at room temperature for 2 min and then centrifuged at 10,000 rpm for 1 min. The EZ-10 spin column was discarded. The collected DNA was stored at -4°C ready for sequencing.

The amplified DNA was sequenced by Illumina genome analyzer sequencing machine (applied Biosystems 3730 xl DNA analyzer). The forward and reverse primer sequences obtained from the sequencing were aligned by Genious R7 program to get the consensus sequences. The consensus sequences were deposited in NCBI GenBank and compared with those available in GenBank via BLAST searches.

Fermentation of the bioactive fungal endophytes and isolation of their secondary metabolites

Endophytic fungi with high antifungal activity were selected for fermentation and testing of their secondary metabolites. Ninety grams of rice were autoclaved twice at 120°C for 15 min in 500 ml Erlenmeyer flasks containing 90 ml of distilled water per flask. Three agar plugs (2 cm × 2 cm) were cut from seven-day-old endophyte cultures on PDA and used to inoculate each flask. One flask, without inoculum, was used as control. After 21 days of incubation at 25°C, 150 ml of methanol was added to each flask and the contents allowed to stand overnight at room temperature. The methanol was filtered and evaporated under reduced pressure to yield the methanol extract which was then subjected to liquid-liquid partitioning with hexane and ethyl acetate. The resulting organic layer was evaporated under reduced pressure to produce the hexane and ethyl acetate extracts.

Testing for chemical constituents of crude fungal endophyte extracts

The endophyte extracts were tested for steroids, terpenoids, saponins, alkaloids using the methods described by Sofowora (1993). Test for steroids was done using Liebermann-Burchard method where 2 ml of acetic acid was added to 0.2 g of each extract and the solution cooled in ice, followed by the addition of conc. H_2SO_4 carefully. Colour development from violet to blue or bluish-green indicated the presence of a steroidal ring that is aglycone portion of cardiac glycoside, while colour change from pink to violet indicated presence of terpenoids whereby the extract was dissolved in ethanol followed by addition of 1 ml of acetic anhydride and conc. H_2SO_4. One gram of each extract was boiled in 5 ml of distilled water and filtered; 3 ml of distilled water was added to the filtrate and shaken vigorously. Formation of Froth indicated

presence of saponins (Sofowora, 1993). Test for flavonoids was done using Shinoda's test (Trease and Evans, 2002) whereby the extracts were dissolved in 1.5 ml of 2% hydrochloric acid followed by addition of 2-3 drops of Mayers reagent. Alkaloids were detected by the formation of a creamy or white precipitate (Sofowora, 1993). Three mililitres of 10% NaOH was added to 2 ml of the different extracts dissolved in distilled water and a change in colour from the original to yellow indicated presence of coumarins (Velavan, 2015). Presence of sterols and triterpenes was tested using Liebermann-Burchard Reaction (Velavan, 2015) whereas that of anthraquinones was done using Borntrager's Test (Sofowora, 1993). Two milliliters of the extracts dissolved in distilled water were added to 2 ml of 2 N hydrochloric acid and ammonia, and formation of a pink red colour that turns blue-violet indicated the presence of anthocyanins (Velavan, 2015). About 10 ml of distilled water was added to 0.5 g of the extract stirred, filtered and 2-3 drops of 1% ferric chloride solution added to the filtrate and formation of a blue-black, green or blue-green precipitate indicated the presence of tannins (Trease and Evans, 2002). Emodins, on the other hand, were tested by adding 2 ml of NH_4OH and 3 ml of Benzene to the extract and their presence detected by formation of a red colour (Savithramma, 2011). Leucoanthocyanins were screened by addition of 5 ml of the aqueous extract to 5 ml of isoamyl alcohol leading to the appearance of red colour in the upper layer.

Bioassay of fungal endophyte crude extracts against *C. lindemuthianum*

Fungal endophytes extracts were subjected to qualitative secondary screening for their antifungal activities using the disc diffusion method with slight modifications, as described by Premlata et al. (2012). Five Agar blocks were cut from actively growing *C. lindemuthianum* culture and transferred into a sterile vial containing 5 ml of sterilized distilled water. The culture blocks were crushed using a sterile glass rod to make a fungal suspension. One milliliter of the fungal suspension was uniformly spread on sterile PDA media in Petri dishes using a sterile cotton swab. From the dried methanol, hexane and ethyl acetate extracts 20 mg was weighed and dissolved in 1ml of DMSO to make a concentration of 20 mg/ml. Sterilized 6 mm blank sensitivity discs were impregnated with 10 µl of the 20 mg/ml concentration of the different extracts and placed at equal distance on the inoculated culture plates. The plates were incubated at 25°C for 7-21 days and inhibition zone diameters measured. The experiments were done in triplicate; Nystatin was used as a positive control and DMSO as the negative control.

Minimum inhibitory concentration (MIC) of bioactive fungal endophyte crude extracts

MIC for the bioactive extracts was determined by testing double fold serial dilutions following the method used by Agbabiaka and Sule (2008) with slight modifications. Methanol extract was not subjected to MIC test due to low inhibition against the test pathogen. Initial concentration of 200 mg/ml (stock solution) was prepared by measuring 400 mg of the hexane and ethyl acetate extracts and dissolving in 2 ml of DMSO. By diluting 1 ml of 200 mg/ml (stock solution) to 2 ml using 1 ml of DMSO, 100 mg/ml concentration was obtained. From 100 mg/ml (stock solution), 1 ml was diluted to 2 ml using 1 ml of DMSO to make a concentration of 50 mg/ml. The aforementioned process was repeated several times to obtain the other concentrations of 25, 12.5 and 6.25 mg/ml. Then 6 mm diameter sterile sensitivity discs were impregnated with 10 µl of the different concentrations and placed at different points on *C. lindemuthianum* inoculated culture plates. After allowing the sample to diffuse for 5 min, the plates were incubated at 25°C for 7 to 21 days. The experiments were done in triplicates; Nystatin was used as a positive control and DMSO as the negative control. Inhibition zone diameters between the pathogen and extracts were measured after 21 days and the averages determined.

RESULTS

Isolation of fungal endophytes and antifungal screening

A total of 37 different fungal endophytes were isolated from the leaves, stem bark and roots of *A. abyssinicus.* Nine of the endophytes (24.3%) were isolated from the leaves, 23 from the bark (62.2%) and five from the roots (13.5%), indicating that a larger percentage of the fungal endophytes inhabit the upper parts of the host plant. Dual culture assay showed that 33 out of 37 fungal endophytes had antagonistic activity against C. lindemuthianum (Figure 1, Table 1). Endophytic fungus *A. hancockii* (Aal - 20) had the highest percentage inhibition zone of 82.6±6.5 against *C. lindemuthianum* followed by Aar-9 (79.7±3.3), Aal-19 (71.7±11.5), and Aar-25 (71.3±1.6). The activity of fungal endophyte Aab-14 and Aar-23 against C. lindemuthianum had no significant difference ($P > 0.05$) as compared to nystatin standard while Aab-36 had a low percentage inhibition of 4.0±3.5 against the test pathogen. Fungal endophytes isolated from the roots of *A. abyssinicus* had the highest percentage inhibition of 58% on average against the test pathogen followed by fungal endophytes isolated from the leaves at 51% and those from the bark having average percentage inhibition of 31.2%.

Molecular identification of fungal endophytes

Four fungal endophytes that showed good antifungal activity against *C. lindemuthianum* were identified using the ITS-rDNA sequence analysis. They included *Aspergillus hancockii, Penicillium christenseniae, Penicillium atrosanguineum* and *Penicillium manginii* (Table 2). Optimal PCR products of the four bioactive isolates were obtained using primer pair ITS1F and ITS4 and the BLAST percentage similarity of the identified fungal endophytes to the sequences in NCBI from the previously identified fungi ranged from 99.66 to 100%.

Bioassay and MIC of endophytes' extracts

Extracts from the isolated fungal endophytes showed varying activity against *C. lindemuthianum* (Table 3) except the crude methanol extract of *P. christenseniae*. *A. hancockii* hexane crude extract gave the highest inhibition of 19.0±1.7 mm at 100% concentration (100 mg/ml) against the test pathogen compared to the Nystatin standard at 48.0±3.5 mm. The ethyl acetate

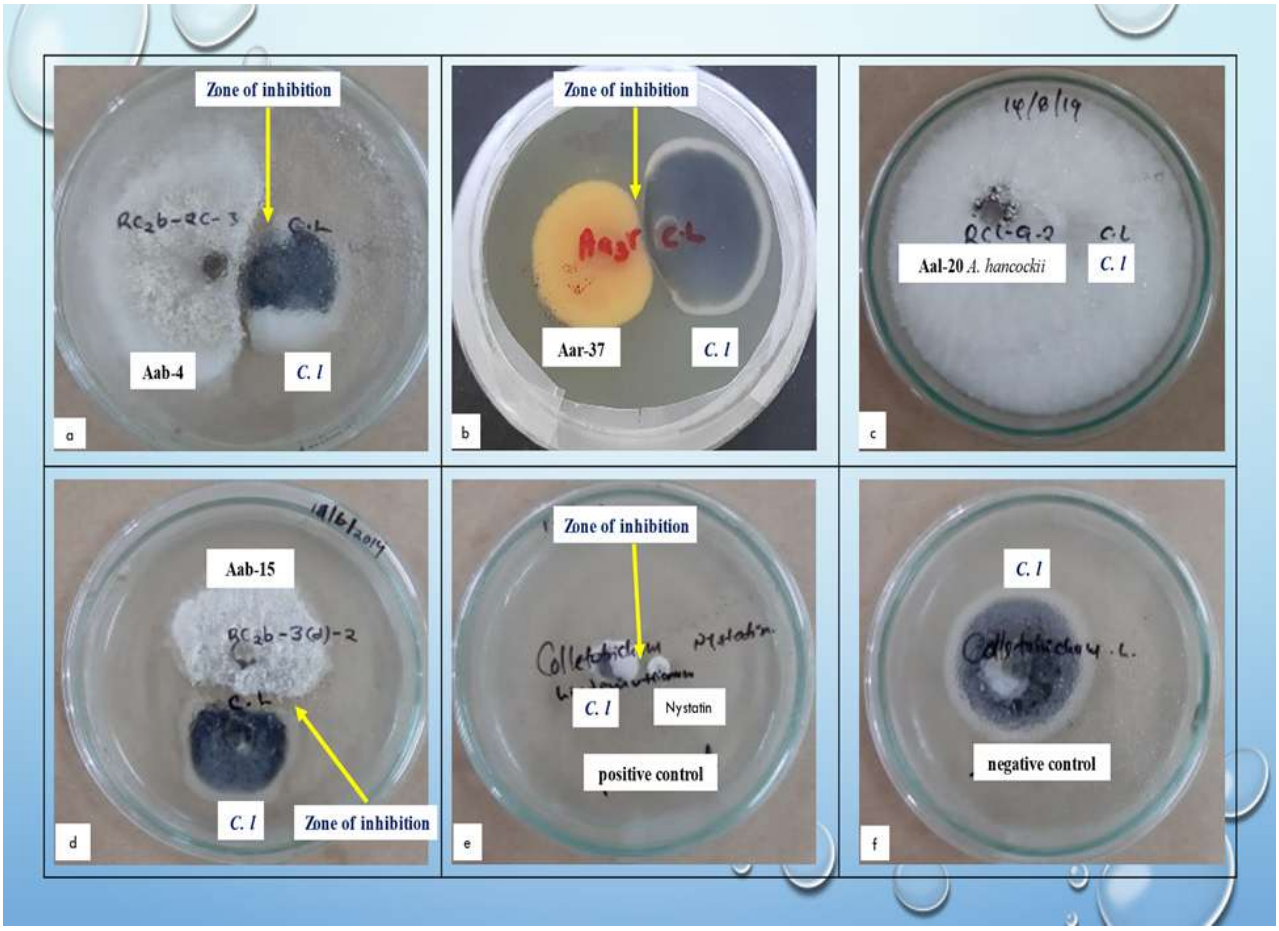


Figure 1. Dual culture test results (a-d) of selected endophytic fungi (Aab-4, Aar-37, Aal-20 and Aab-15) from *Allophyllus abyssinicus* against *Colletotrichum lindemuthianum* (*C. l*), e – positive control using nystatin against the pathogen and f – negative control with the pathogen left to grow on its own.

crude extract of *A. hancockii* had a similar inhibition (8.0±1.7 mm) as its crude methanol extract (8.0±1.0 mm) but both exhibited a lower inhibition compared to Nystatin. Both hexane (8.3±0.6 mm) and ethyl acetate (10.3±0.6 mm) crude extracts of *P. christenseniae* had some inhibition against *C. lindemuthianum* which was lower than Nystatin. Hexane extracts from both fungal endophytes had a good inhibition against the test pathogen, with the methanol extract of *P. christenseniae* lacking activity. The activity of *P. christenseniae* and *A. hancockii* extracts corresponds to the data obtained in the dual culture assay, with *A. hancockii* having higher inhibition.

A. hancockii hexane extract had the highest inhibition of 19.0±1.7 mm (100% concentration) compared to all the other extracts from the two fungal endophytes with *A. hancockii* ethyl acetate crude extract having the lowest inhibition of 8.0±1.7 mm (Table 4). These inhibition results of *A. hancockii* are in line with both the dual culture and bioassay screening of its hexane extracts against *C. lindemuthianum.* On average, *P. christenseniae* gave a higher inhibition against the test pathogen compared to *A. hancockii* at lower concentrations, with both fungal endophytes having lower inhibition compared to the standard Nystatin. *P. christenseniae* hexane extract had a lower inhibition of 8.3±0.6 mm compared to its ethyl acetate extract at 10.3±0.6 mm. Minimum inhibitory concentration for the extracts was determined as 0.0625 mg/ml ml and 0.313 mg/ml for both *A. hancockii* and *P. christenseniae* hexane and ethyl acetate crude extracts respectively. At 6.25% concentration, *A. hancockii* hexane extracts gave inhibitions of 6.3±3.8, while *P. christenseniae* gave inhibitions of 8.0±2.0 and 7.3±1.5 mm (hexane and ethyl acetate extracts, respectively).

Chemical constituents in crude extracts of *Allophyllus abyssinicus* fungal endophytes

Steroids were only present in *P. christenseniae* methanol and ethyl acetate crude extracts but absent in *A.*

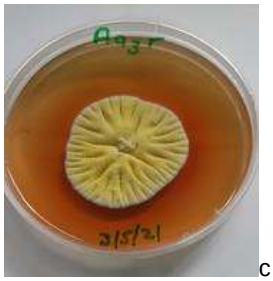
Table 1. Inhibition of different *Allophyllus abyssinicus* fungal endophytes against *Colletotrichum lindemuthianum*.

Fungal endophyte	Percentage inhibition (n=3)
	C. lindemuthianum
Aab-1	20.1±3.4^{ab}
Aab-2	25.4±3.4^{abc}
Aab-3	60.1±9.0^{fghi}
Aab-4	38.4±8.2^{bcd}
Aal-5	40.2±4.8^{bcdef}
Aal-6	34.1±2.5^{bcd}
Aab-7	22.5±4.5^{ab}
Aal-8	44.9±2.5^{cdef}
Aar-9 (*P. christenseniae)*	79.7±3.3^{ij}
Aal-10	43.5±13.2^{cdef}
Aab-11	10.9±5.8^{a}
Aab-12	26.8±2.5^{abc}
Aab-13	36.9±2.0^{bcd}
Aab-14	65.2±9.5^{ghij}
Aab-15	33.3±5.0^{bcd}
Aal-16	59.4±5.5^{efgh}
Aal-17	39.9±7.0^{bcde}
Aal-18	47.8±5.8^{defg}
Aal-19	71.7±11.5^{hij}
Aal-20 (*A. hancockii*)	82.6±6.5^{j}
Aab-21	62.2±22.3^{ghi}
Aab-22	63.9±2.8^{ghi}
Aar-23 *(P. manginii)*	66.7±5.6^{ghij}
Aab-24	59.3±4.2^{efgh}
Aar-25 *(P. atrosanguineum)*	71.3±1.6^{hij}
Aar-26	44.1±10.9^{cdef}
Aab-27	37.4±12^{bcd}
Aab-28	42.6±5.8^{cdef}
Aab-29	35.9±7.6^{bcd}
Aab-30	6.0±2.5^{a}
Aab-31	27.3±14.6^{abc}
Aab-32	6.0±1.2^{a}
Aab-33	6.0±3.3^{a}
Aab-34	47.7±17.6^{defg}
Aab-35	6.0±2.7^{a}
Aab-36	4.0±3.5^{a}
Aar-37	31.3±7.5^{bcd}
NYSTATIN	63.5±1.8^{ghij}

Within a column, fungal endophytes sharing the same letter(s) are not significantly different in antagonism against *Colletotrichum lindemuthianum* while those with different letters are significantly different (α =0.05, Tukey's test). The inhibition zone values are the mean of three replicates ±S.D. of the mean. Aal = *A. abyssinicus* leaves; Aar =*A. abyssinicuss* roots; Aab =*A. abyssinicus* bark.

hancockii. Saponins and alkaloids were present in both endophyte extracts, while flavonoids were found only in *A. hancockii* methanol and hexane extract. Coumarins, sterols and triterpenes were also only present in *A. hancockii* whereas Leucoanthocyanins were only found in *P. christenseniae.* From the chemical constituent screening of these two fungal endophytes extracts there were no traces of terpenoids, anthraquinones,

Table 2. Identification of fungal endophytes from *Allophylus abyssinicus* using molecular techniques.

Fungal endophyte isolate	Molecular identification		
	Similarity %	Accession number	Similarity with
 a	99.6	MT530175.1	*P. christenseniae* (Aar - 9)
 b	99.66	NR154725.1	*A. hancockii* (Aal - 20)
 c	100	MH857072.1	*P. atrosanguineum* (Aar - 25)
 d	100	MH858641.1	*P. manginii* (Aar - 23)

anthocyanins, tanins, and emodins (Table 5).

DISCUSSION

Isolation and antifungal activity of *A. abyssinicus* fungal endophytes and their extracts

Different fungal endophytes were isolated from the medicinal plant *A. abyssinicus* fresh stem bark, roots and leaves. This isolation results are in agreement with the study by Carbungco et al. (2017) whereby 24 different fungal endophytes were isolated from Moringa oleifera leaves indicating that different fungal endophytes can be isolated from different parts of a plant. The results also confirm that endophytes are present in all plant tissues of the host plant and are rich sources of active biomolecules as reported by Kusari et al. (2012). Very little research has been done on isolation and diversity of endophytes from *A. abyssinicus* but recently *Moniliophthora*

Table 3. Inhibition zones of selected bioactive fungal endophyte crude extracts against *Colletotrichum lindemuthianum*.

Crude fungal endophyte extract	Inhibition diameter in mm (n=3) Bioassay
A. hancockii MeoH	8.0±1.0[a]
A. hancockii Hexane	19.0±1.7[b]
A. hancockii EtoAc	8.0±1.7[a]
P. christenseniae MeoH	6.0±0.0[a]
P. christenseniae Hexane	8.3±0.6[ab]
P. christenseniae EtoAc	10.3±0.6[ab]
Nystatin	48.0±3.5[c]

Within a column, fungal endophytes extracts sharing the same letter(s) are not significantly different in inhibition against *Colletotrichum lindemuthianum* while those with different letters are significantly different (α =0.05, Tukey's test). The inhibition zone values are the mean of the triplicates ±S.D. of the mean. MeoH: Methanol and EtoAc: ethyl acetate.

Table 4. Inhibition zones and MIC of selected bioactive fungal endophyte crude extracts against *Colletotrichum lindemuthianum*

	100%	50%	25%	12.5%	6.25%	3.125%	(MIC mg/ml) 0.625%
Nystatin		+ve=64					-ve=0
A. hancockii							
Hexane	19.0±1.7	10.3±3.5	8.0±2.6	6.7±0.7	6.3±3.8	6.0±0.5	0.6256
EtoAc	8.0±1.7	6.7±0.6	6.0±3.5	6.0±3.5	6.0±0.0	6.0±0.0	5.0006
P. christenseniae							
Hexane	8.3±0.6	8.0±2.0	8.0±1.0	8.0±1.5	8.0±2.0	6.8±1.5	0.313
EtoAc	10.3±0.0	8.7±1.5	8.0±1.7	7.7±2.1	7.3±1.5	6.2±1.9	0.313

perniciosa a non-pathogenic endophytic biotype was isolated from the bark of *A. edulis* in research by Lisboa et al. (2020). Most of the fungal endophytes isolated from *A. abyssinicuss* were situated above the ground with 9 from the leaves, 23 from the stem bark compared to those isolated from the roots which were 5 and all had different characteristics. Results obtained from this isolation are also in agreement with those reported by Alvin et al. (2014) where every plant species hosts various endophytes depending on their habitat and different plant parts.

Different fungal endophytes isolated from *A. abyssinicus* exhibited varying activity against *C. lindemuthianum* in the dual culture assay, with *A. hancockii* isolated from the leaves having the highest percentage inhibition (82.6±6.5) against the test phytopathogen. It is worth noting that fungal endophytes isolated from the roots had the highest percentage inhibition (58%) on average against the test phytopathogen followed by those from the leaves (51%) and bark at (31.2%). *P. christenseniae* isolated from the roots had the second highest percentage inhibition (79.7±3.3). This result corresponds to Korejo et al. (2014) findings where endophytic *Penicillium* species exhibited significant antifungal activity against *Fusarium solani, Fusarium oxysporum* and *Rhizoctonia solani*. *Penicillium christenseniae* SD.84 isolated from the soil had antimicrobial activity against several bacterial and fungal pathogens *in vitro* as reported by Wang et al. (2022). Both *A. hancockii* and *P. christenseniae* have not been previously screened against *C. lindemuthianum*.

Crude extracts from the two most active fungal endophytes, *P. christenseniae* and *A. hancockii* exhibited antifungal activity against the test pathogen in the bioassay tests. The fungal endophyte *P. christenseniae* hexane extract having an inhibition of 8.3±0.6 100 mg/ml concentration against *C. lindemuthianum* results are in line with the findings of Du Toit (2020) that reported *Penicillium* isolates from the tubers of *Pelargonium sidoides* having antifungal activity against *F. oxysporum* f. sp. *cubense. Aspergillus hancockii* hexane extract had an of inhibition of 19.0±1.7 mm (100 mg/ml) against *C. lindemuthianum* which is in agreement with the findings of Hashem et al. (2022) that reported the endophytic *Aspergillus terreus* isolated from *M. oleifera* leaves to have antifungal activity against *Rhizopus oryzae*, *Mucor*

Table 5. Chemical constituents of crude fungal endophytes' extracts from *Allophyllus abyssinicus*.

Chemical constituent	*A. hancockii* extract			*P. christenseniae* extract		
	Meoh	Hexane	EtoAc	Meoh	Hexane	EtoAc
Steroids	-	-	-	+	-	+
Terpenoids	-	-	-	-	-	-
Saponins	+	+	+	+	-	+
Flavonoids	+	+	-	-	-	-
Alkaloids	+	+	+	+	+	-
Coumarins	+	-	+	-	-	-
Sterols and tri terpenes	+	+	+	-	-	-
Anthraquinones	-	-	-	-	-	-
Anthocyanins	-	-	-	-	-	-
Tanins	-	-	-	-	-	-
Emodins	-	-	-	-	-	-
Leucoanthocyanins	-	-	-	+	-	+

racemosus and *Syncephalastrum racemosum*, with its ethyl acetate crude extracts (10 mg/ml) inhibitions being 20, 37, and 18 mm, respectively. The ethyl acetate and hexane crude extracts of the two endophytes were active against *C. lindemuthianum* and their mean inhibitions were statistically different compared to the Nystatin standard, with *A. hancockii* hexane crude extract being statistically lower than Nystatin and *P. christenseniae* hexane and ethyl acetate crude extracts being statistically lower than the Nystatin mean inhibition.

Antifungal activity of *A*. *hanckockii* against the test pathogen is in line with the results obtained by Nirmal et al. (2018) whereby compounds isolated from *Aspergillus banksianus* had antimicrobial activities and since *A*. *hanckockii* belongs to the same genus as *A. banksianus*. Bolaamphiphilic antifungal compounds, the burnettramic acids were isolated from *Aspergillus burnettii* in research by Li et al. (2019); this antifungal activity has similarity as those of *A. hancockii* against *C. lindemuthianum*. In another study, compounds isolated from *A. burnettii* had antimicrobial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Candida albicans* and *Saccharomyces cerevisiae* in the bioassay screening (Gilchrist et al., 2020) indicating that extracts from fungal endophytes in the genus *Aspergillus* have antimicrobial properties. Antifungal activity of *P*. *christenseniae* is in line with the findings of Wang et al. (2022) indicated that secondary metabolites isolated from this fungus had antimicrobial activity against *S. aureus* and methicillin-resistant *S. aureus*.

MIC of *A. hancockii* were determined as 0.625 mg/ml, 5.000mg/ml and gave inhibitions of 6.3±3.8 mm and 6.7±0.6 mm for the hexane and ethyl acetate extracts, respectively against the test pathogen. At MIC of 0.313 mg/ml *P. christenseniae* hexane extract gave an inhibition of 6.8±1.5 mm. *A. hancockii* hexane extract gave the highest inhibition of 19.0±1.7mm at 100% concentration compared to all the other extracts from the two fungal endophytes with the lowest inhibition obtained from *A. hancockii* ethyl acetate crude extract at 6.0±0.0 mm at 6.25% concentration. This result indicates that the inhibition results of *A. hancockii* are in line with both the dual culture and bioassay screening of its hexane extracts against *C. lindemuthianum.* On average *P. christenseniae* gave a higher inhibition against the test pathogen compared to *A. hancockii* under lower concentrations 6.25%, with both fungal endophytes having very low inhibition compared to the standard Nystatin at 64 mm. Under lower concentrations 6.25% *P. christenseniae* hexane extract gave a higher inhibition diameter of 8.0±2.0 mm compared to its ethyl acetate extract at 7.3±1.5 mm. Results obtained in this study are in line with the findings of Intaraudom et al. (2013) whereby picolinic acid derivatives isolated from a *Penicillium* spp*.* had antifungal activity at MIC values of 1.5 and 3.7 µg/ml. Hexane extracts from both fungal endophytes gave higher inhibition against the pathogens compared to their ethyl acetate extracts. This study, therefore, demonstrates that extracts from the two fungal endophytes isolated from *A. abyssinicus* can be used in agriculture to control common bean anthracnose.

Identification of the fungal endophytes

Out of the 37 fungal endophytes isolated, four that were highly active against *C. lindemuthianum* in the dual culture assay were identified using molecular techniques and they all belonged to the phylum Ascomycota. These results are in agreement with a study by Kirk et al. (2008) that reported Ascomycota as the largest phylum under kingdom Fungi with over 64,000 species and referred to as sac fungi with septate hyphae. Diversity of these fungal endophytes in various plants is normally influenced

by environmental factors, the host and its genetic constitution according to Chen et al. (2010) which is in line with the results obtained in this study whereby different endophytes were isolated from different parts. A lot of endophytes are isolated from this phylum due to their ability to produce ascospores that confer resistance against other microbes under unfavorable environments (Goveas et al., 2011); this is in agreement with the identification results obtained where the bioactive endophytes identified were Ascomycetes.

Three of the isolated active fungal endophytes belonged to the genus *Penicillium* while one belonged to the genus *Aspergillus*. A study by Visagie et al. (2014) indicated *Penicillium* as one of the largest genera in kingdom Fungi, with more than 400 species, distributed globally which is supportive of the results obtained in this study whereby three of the identified endophytes were *Penicillium* spp. *A. hanckockii* isolated in this study has also been previously isolated from soil in peanut fields and other substrates in southeast Australia according to Pitt et al. (2017). The fungus *P. christenseniae* isolated in this research was also isolated from meat products and spices as *P. christenseniae* isolate E20408 in research done by Abd El-Tawab et al. (2020), while *Penicillium atrosanguineum* was isolated by Korur et al. (2019) from forest soil using Waksman's 'Soil Dilution Method' in the study of diversity of *Penicillium* spp. *Penicillium manginii* isolated in this study was isolated as an endophytic fungus (*P. manginii* YIM PH30375), living inside the elder root of *Panax notoginseng* medicinal plant in China in a study by Pei et al. (2015).

Screening of chemical constituents of the fungal endophyte extracts

Various secondary metabolites were present in different fungal endophytes isolated from *A. abyssinicus* in this study. These included; steroids, saponins, flavonoids, alkaloids, coumarins, sterols, tri-terpenes and leucoanthocyanins. The genus *Allophylus* contains medicinally important phytochemicals such as phenols, alkaloids, saponins, sesquiterpenes, steroids, anthraquinones among others isolated from the different plant parts and its endophytes. Presence of steroids, saponins, alkaloids and leucoanthocyanins in *P. christenseniae* is in agreement with a study done by Domracheva et al. (2021) which indicate that *Penicillium* spp. contains active natural products and this secondary metabolites are involved in inhibitory activities against microbes (Kozlovskii et al., 2013). Presence of alkaloids in *P. christenseniae* crude extracts is in line with the findings of Wang et al. (2022) whereby, two new quinolone alkaloid enantiomers, (*Ra*)-(-)-viridicatol (1) and (*Sa*)-(+)-viridicatol (4) and seven other known compounds were isolated from *P. christenseniae* SD.84. The presence of steroids in *P. christenseniae* ethyl acetate crude extract could have contributed to the inhibition of 10.3±0.6 mm, with the difference in inhibition being probably a result of synergy of the chemical constituents present in each extract. This is in line with the report of Chavan and Gaikwad (2016) that indicates the presence of steroids in *A. edulis* which is in the same genus as *A. abyssinicus*.

The presence of flavonoids in *A. hancockii* hexane extract could have contributed to the high inhibition of 19.0±1.7 mm compared to the other extracts. The presence of coumarins, sterols and triterpenes in *A. hancockii* could be the reason why this fungal endophyte was very active in the dual culture, extract bioassay and minimum inhibitory concentration tests against the test pathogen. A study by Chavan and Gaikwad (2016) reported the presence of flavonoids, coumarins, sterols and triterpenes in *A. edulis* which were also present in *A. hancockii* and could have produced a synergistic effect that increased inhibition of *C. lindemuthianum in vitro* since these compounds are absent in *P. christenseniae* which was less active compared to *A. hancockii.* The fungal endophyte *A. hancockii* was reported to yield a total of 69 different types of secondary metabolites, one of them being a novel metabolite called dehydroterrestric acid in a study by Pitt et al. (2017); others isolated included Kojic acid, Onychocin A and B, Speradine F, 7-hydroxytrichothecolon among others which are in line with the findings of this research that various compounds can be isolated from a specific endophyte. A combination of all these different metabolites produced by this fungal endophyte may confer antifungal activity against *C. lindemuthianum* suggesting that they may be toxic or lethal to the pathogen and can be used in management of bean anthracnose.

Results from this study indicate that bioactive secondary metabolites from *A. abyssinicuss* fungal endophytes can be an effective alternative in the control of bean anthracnose due to the fact that biocontrol pose no dangers to the environment and human beings. It is cheaper, compatible with other disease management methods and are highly biodegradable. Use of this secondary metabolite in the disease control will lead to increased production of beans of high quality, thereby increasing their marketability, contribute in reducing food insecurity and hidden hunger to the ever-increasing human population.

Conclusion

This study proved that the medicinal plant *A. abyssinicus* hosts different strains of endophytic fungi that can be used effectively to control *C. lindemuthianum*. The data obtained indicated that these fungal endophytes contain secondary metabolites with antifungal activity against *C. lindemuthianum* and, therefore, can be used to manage

bean anthracnose and boost bean production globally.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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